J. Mol. Biol. (1968) **32**, 159–168

Structure of the Replicative Form of Bacteriophage ϕX174

V.† Interconversions between Twisted, Extended and Randomly Coiled Forms of Cyclic DNA

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(*Received 17 August 1967, and in revised form 17 October 1967*)

As is already known, double-stranded DNA of bacteriophage ϕX174 occurs in a twisted cyclic double helix (21 s). The two strands making up the double helix are separately continuous. The tertiary twists in the molecule are released by the introduction of single-strand chain breaks by pancreatic DNase. The product formed is an extended cyclic helix (17 s). A third form of double-stranded ϕX174 DNA, known as coiled cyclic double-stranded DNA (40 s in M-NaCl at neutral pH) is obtained upon alkaline denaturation of the twisted cyclic helix.

In this paper, quantitative proof is given that the conversion of the twisted cyclic helix to the extended cyclic form is induced by only one single and random chain break. The conformational change is accompanied by a twofold decrease in biological activity. The further biological inactivation of the extended ring form occurs at a rate which suggests that approximately one out of 20 single-strand chain breaks in double-stranded DNA is lethal.

The introduction of single-strand chain breaks into the cyclic coil (40 s) leads to the spontaneous restoration of the helical structure. An extended cyclic helix (17 s) is formed as indicated by ultracentrifugal analysis and electron microscopy. It has been shown that the previously reported but unexplained high resistance to ultraviolet inactivation of the denatured cyclic coil is due to host-cell reactivation. The required DNA double helix may arise from the cyclic coil by the action of nucleases known to be involved in host-cell reactivation.

1. Introduction

Several viral DNA's including those of polyoma virus (Vinograd, Lebowitz, Radloff, Watson & Laipis, 1965), rabbit and human papilloma virus (Crawford, 1965) and the vegetative forms of bacteriophages ϕX174 (Jansz & Pouwels, 1965; Burton & Sinsheimer, 1965; Roth & Hayashi, 1966; Jaenisch, Hofschneider & Preuss, 1966)

† Paper IV in this series is Jansz, Pouwels & Schiphorst (1966).

and λ (Bode & Kaiser, 1965; Young & Sinsheimer, 1964) consist of a twisted cyclic double helix (Plate I(a)). There are two essential features of this structure.

(1) The two strands making up the double helix are separately continuous.

(2) Tertiary twists are superimposed on the turns of the double helix. These tertiary twists in cyclic DNA may arise from an excess or deficiency of turns in the Watson–Crick double helix, as compared to linear DNA. The continuity of each of the two single strands in the cyclic double helix is required for the preservation of the tertiary structure. Introduction of single-strand chain breaks (Burton & Sinsheimer, 1965; Jansz & Pouwels, 1965) converts the twisted cyclic double helix into an extended circular double helix (Plate I(b)).

The interlocking of the two strands in the twisted circle prevents the strands from coming apart under conditions of denaturation which lead to strand separation of linear DNA. Exposure of the twisted circle to increasing conditions of denaturation leads to coiling which becomes irreversible at a critical point, and the twisted circle is converted into a randomly coiled cyclic double-stranded DNA (Pouwels & Jansz, 1964).

This paper describes studies on the interconversions between the twisted cyclic helix (21 S), the extended cyclic helix (17 S) and the cyclic coil (40 S in M-NaCl at neutral pH) using the double-stranded DNA of bacteriophage φX174. In these studies it was of particular advantage that all the forms of double-stranded φX174 DNA as well as the single-stranded DNA of the phage are endowed with biological activity, i.e. are capable of infecting spheroplasts, leading to the induction of φX174 phage.

2. Materials and Methods

(a) *Bacteriophage and bacterial strains*

Escherichia coli C and bacteriophage φX174 were obtained from Dr R. L. Sinsheimer.

Spheroplasts were prepared either from *E. coli* K12, or from a mutant which has lost the property of host-cell reactivation (*E. coli* K12 *hcr*$^-$).

(b) *Preparation of DNA*

Single-stranded DNA of bacteriophage φX174 was obtained by extraction with phenol followed by Sephadex filtration (Jansz, van Rotterdam & Cohen, 1966) of phage batches containing approximately 10^{14} viable phage/ml. Phage was prepared according to a modified (Jansz, van Rotterdam & Cohen, 1966) procedure of Sinsheimer (1959*a*,*b*).

Double-stranded φX174 DNA was prepared according to a recently developed method (Jansz, Pouwels & Schiphorst, 1966).

Coiled cyclic double-stranded φX174 DNA was obtained by treatment of twisted cyclic DNA in 0·6 M-NaCl–0·1 M-sodium phosphate at pH 12·5 for 10 min at 20°C followed by neutralization of the solution. In order to free twisted cyclic φX174 DNA of the extended ring form, the solution in 0·6 M-NaCl–0·1 M-sodium phosphate is brought to pH 11·5 at 20°C and incubated at this pH for 2 min and then neutralized. During this procedure, the extended ring form is converted to single-stranded DNA, whereas the twisted form remains unchanged. The solution is filtered using a 0·2 μ MF14 filter (Membranfilter Gesellschaft, Göttingen, Germany). Denatured DNA remains on this filter, whereas native DNA passes through at high salt concentration.

The concentration of single-stranded DNA molecules was calculated from the absorbance at 260 mμ using the relation $1{\cdot}32 \times 10^{13}$ molecules/ml. (36 μg DNA/ml.) per absorbance unit at 260 mμ measured in 0·2 M-NaCl at 25°C, pH 7·0 (Guthrie & Sinsheimer, 1963). For double-stranded DNA, an absorbance unit at 260 mμ corresponds to 50 μg DNA/ml. and 9×10^{12} molecules/ml.

(c) *Digestion of DNA by DNase*

Dilutions of DNase (Nutritional Biochemical Corporation, twice crystallized) were made through 0·1% bovine serum albumin (Armour) in 0·01 M-phosphate buffer (pH 7·0) in the cold. To obtain the final concentration of enzyme, 1/20 vol. of the appropriate DNase dilution was added to a solution of DNA in 0·01 M-phosphate and 2 mM-$MgCl_2$ at 20°C.

The reaction was terminated by the addition of sodium citrate to a final concentration of 0·01 M. Samples which were to be analysed at alkaline pH were dialysed against 0·001 M-citrate in order to prevent coprecipitation of DNA with $Mg(OH)_2$.

(d) *Sedimentation*

Sedimentation analyses were performed in a Spinco model E analytical ultracentrifuge using the band sedimentation technique (Vinograd, Bruner, Kent & Weigle, 1963). Analyses were performed either in 2 M-NaCl at neutral pH or in CsCl ($\rho = 1{\cdot}35$) containing 0·01 M-phosphate at pH 12·5 (Vinograd, Morris, Davidson & Dove, 1963). For sedimentation at neutral pH, an AN-E rotor was used; for sedimentation at alkaline pH, an AN-D rotor was used.

(e) *Biological activity of ϕX174 DNA*

DNA was incubated with spheroplasts according to Guthrie & Sinsheimer (1960) and phage titres were determined after lysis of the spheroplasts, 3 hr after the addition of DNA.

(f) *Ultraviolet irradiation*

Ultraviolet irradiation was performed by illumination of DNA samples in 0·01 M-phosphate (pH 7·0) with a low-pressure mercury tube (Philips, 30 w, T.U.V.).

(g) *Electron microscopy*

Specimens were prepared by a slightly modified Kleinschmidt technique (Kleinschmidt, Lang, Jackerts & Zahn, 1962; Borst, van Bruggen, Ruttenberg & Kroon, 1967).

The solutions used for the spreading procedure contained about 4 μg DNA/ml. in 1·3 M-ammonium acetate and 0·01% cytochrome *c*. A Petri dish covered with a fresh sheet of Parafilm was used as a trough that was filled with 0·1 M-ammonium acetate. After spreading, the specimens were shadowed under rotation (speed 3000 rev./min) with 8 mg platinum at a distance of 5 cm and an angle of 8°.

3. Results

(a) *Biological activity*

The infectivities of different forms of ϕX174 DNA have been measured previously (Pouwels & Jansz, 1964; Sinsheimer, Lawrence & Nagler, 1965), but a quantitative comparison of the infectivities of the purified forms has not been reported. In Fig. 1 the concentration–activity relationships of double, single and coiled double-stranded ϕX174 DNA are compared. It can be seen that single-stranded DNA is approximately ten times as active as double-stranded DNA over the entire range of DNA concentrations, whereas the activity of coiled DNA is in between. The three curves all level off at DNA concentrations above 10^{11} molecules per ml., which is probably due to the saturation of all competent spheroplasts with DNA.

The tenfold difference in activity between single and native double-stranded DNA is probably due to a greater efficiency of the spheroplasts in taking up single- as compared to double-stranded DNA, rather than to a difference in the initiation of phage replication. This is supported by the fact that the activity of double-stranded DNA in the coiled form is closer to that of single-stranded DNA.

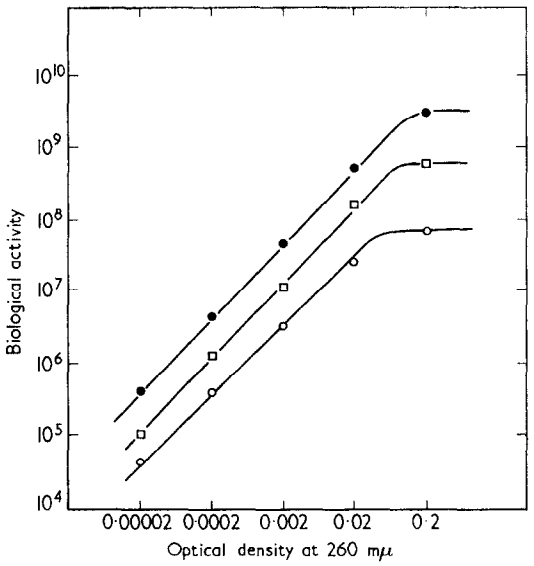


FIG. 1. Relationship between the optical density of ϕX174 DNA and biological activity (number of plaque-forming units produced in spheroplasts).
—●—●—, Single-stranded DNA; —□—□—, coiled double-stranded DNA; —○—○—, double-stranded DNA.

It has been shown earlier (Jansz, Pouwels & Rotterdam, 1963; Yarus & Sinsheimer, 1964) that the difference in ultraviolet sensitivity between single- and double-stranded DNA of ϕX174 is largely due to the fact that within the host cell ultraviolet lesions can be repaired in double- but not in single-stranded DNA. This is borne out by the observation that double-stranded DNA acquires a radiosensitivity which is very near that of single-stranded DNA when assayed on spheroplasts of *E. coli* K12 *hcr*$^-$, a strain that has lost the power to repair ultraviolet lesions. It has been observed earlier (Pouwels & Jansz, 1964; Sinsheimer *et al.*, 1965) that the ultraviolet sensitivity of the denatured cyclic coil is that of helical double-stranded ϕX174 DNA (measured on *hcr*$^+$ spheroplasts). This is due to host-cell reactivation as shown in Fig. 2, where the ultraviolet survival curves of single-stranded, double-stranded and coiled double-stranded ϕX174 DNA are compared using *hcr*$^+$ and *hcr*$^-$ spheroplasts.

It can be seen that the host cell is able to repair ultraviolet lesions in coiled DNA and helical double-stranded DNA with virtually equal efficiency. The required helical structure in coiled DNA might be induced (see sections (c) and (d)) during host-cell reactivation, which is known to involve the action of nucleases.

(b) *Effect of DNase on twisted cyclic double-stranded DNA*

It has been shown earlier (Jansz & Pouwels, 1965) that the initial chain scissions by pancreatic DNase in twisted cyclic ϕX174 DNA change the sedimentation coefficient from 21 to 17 s. This is due to a change in the conformation of the molecule from a twisted to an extended ring structure as indicated by electron microscopy (Roth & Hayashi, 1966; Jaenisch *et al.*, 1966).

It is the purpose of the present section to determine the number of enzymic chain breaks per molecule that are required for the initiation of this conformational change,

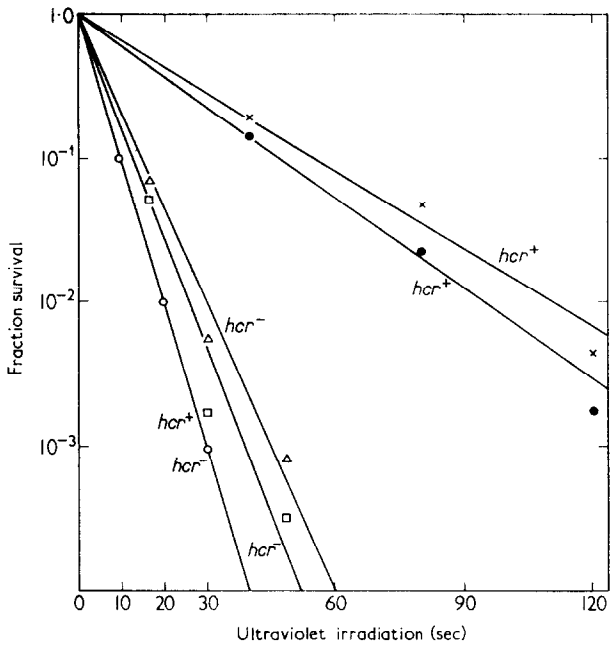


FIG. 2. Survival of ϕX174 DNA after ultraviolet irradiation assayed on *hcr*$^+$ and *hcr*$^-$ spheroplasts.
—○—○—, Single-stranded DNA; —×—×— and —△—△—, double-stranded DNA; —●—●— and —□—□—, coiled double-stranded DNA.

and to investigate whether these breaks occur at a specific place in the molecule or in a random fashion. Also the effect of these chain breaks on the biological activity is investigated.

Twisted ϕX174 DNA (21 S component, 95 μg/ml.) was treated for different lengths of time with DNase (0·00005 μg/ml.) as described under Materials and Methods. The samples were analysed in the analytical centrifuge in 2 M-NaCl at pH 7·0. Decreasing amounts of 21 S and increasing amounts of 17 S component were observed as the duration of DNase action proceeds.

In Fig. 3 the fraction of 21 S component *versus* time of DNase action is plotted on a semi-logarithmic scale. In agreement with previously reported experiments (Jansz & Pouwels, 1965), a strictly exponential relationship is obtained, suggesting a "one-hit" mechanism.

However, this does not exclude the occurrence of a short-lived intermediate, and the possibility exists that the conversion may require two or more consecutive single-strand chain breaks.

In order to investigate this quantitatively, the number of single-strand chain breaks in the extended ring form was estimated as a function of the conversion of 21 S into 17 S as follows.

The samples (Fig. 3) were analysed in CsCl at pH 12·5 in the analytical ultracentrifuge. Under these conditions, the twisted form (21 S) is converted into the coiled form (53 S). The extended circular form which contains chain breaks is resolved into two species sedimenting at 18 S and 16 S (Fiers & Sinsheimer, 1962), representing

single-stranded circular and linear molecules, respectively (Plate II). The ratio of 16 S to 18 S particles depends on the number of single-strand chain breaks required to induce the conversion of the twisted into the extended circle. Theoretical relations between the ratio of 16 S to 18 S particles and the fractional conversion of the twisted into the extended form were computed from the Poisson equation, assuming that either one (lower curve) or two (upper curve) chain breaks per molecule are required (Fig. 4). The upper curve takes account of there being a probability of one break on each strand as well as two breaks on one strand. The experimental values in Fig. 4 fit the lower curve, indicating that only one random break is required for the con-

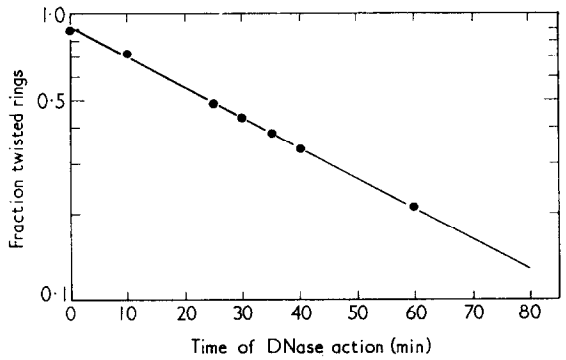


FIG. 3. Fraction of twisted rings *versus* time of DNase action. DNA concentration: 95 μg/ml. DNase concentration: 0·00005 μg/ml.
Ultracentrifugal analysis was performed in 2 M-NaCl in an AN-E rotor at 44,770 rev./min.

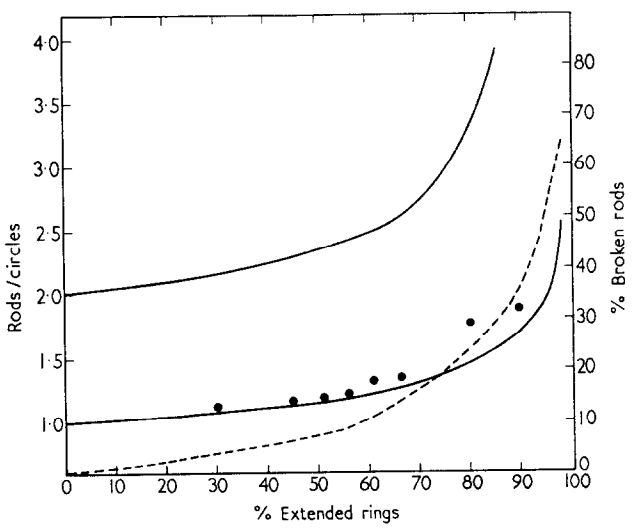


FIG. 4. The theoretical relation between the ratio of single-stranded rods to single-stranded circles (left-hand ordinate) as a function of the conversion of twisted into extended ring form.
The theoretical relations were computed from the Poisson equation, assuming that one (lower curve) or two chain breaks per molecule (upper curve) are required for the conversion. The dotted line represents the theoretical fraction of broken rods (right-hand ordinate) as a function of the percentage extended rings. The experimental values are represented by dots. The ratio rods/circles was determined using the band sedimentation technique (see Plate II).

formational change of twisted cyclic ϕX174 DNA into the extended ring form. The fraction of broken rods arising at one break per molecule is neglected in these considerations since this fraction is small (dotted line, Fig. 4) in the range of the experimental values.

The biological inactivation of twisted cyclic DNA (Fig. 5) takes place in two phases: an initial fast decrease in biological activity is followed by a slower decrease in the later stages. The initial fast decrease in activity is caused by the first enzymic chain break, which converts the twisted circular DNA into the open ring form. This statement is based on the fact that the initial decrease in activity parallels the conversion of 21 s into 17 s component (Fig. 5) as determined by ultracentrifugal analysis. This result is not unexpected, since the two forms of DNA may differ in the efficiency in which they infect spheroplasts. By extrapolation of the final slope of the survival curve, it may be estimated that the extended cyclic form is approximately half as active in the spheroplasts assay as the twisted cyclic form.

It can be assumed that the biological inactivation after 20 minutes (Fig. 5) follows an exponential course. This is particularly evident in experiments using higher DNase concentrations, where exponential inactivation over three orders of magnitude has been demonstrated.

It can be seen that the dose of DNase required to inactivate the extended circular form is approximately 20 times greater than that required for the conformational change (Fig. 5), indicating that one-twentieth of the single-strand chain breaks in double-strand ϕX174 DNA are lethal.

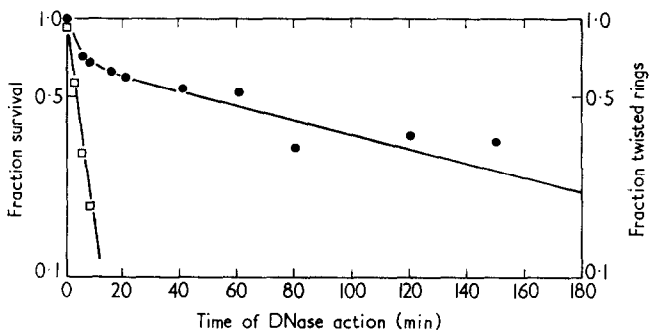


FIG. 5. The biological inactivation (left-hand ordinate) of twisted cyclic DNA by DNase (—●—●—).
Fraction of twisted rings (right-hand ordinate) *versus* time of DNase action in minutes (—□—□—). This fraction was determined as in Fig. 3. DNA concentration: 50 μg/ml. DNase concentration: 0·0002 μg/ml.

(c) *Effect of DNase on the cyclic coil*

Coiled double-stranded ϕX174 DNA (40 μg/ml.) was incubated with DNase (0·0002 μg/ml.) for different lengths of time, as described under Materials and Methods.

The samples were subjected to biological assay and sedimentation analysis. Ultracentrifugal analysis in 2 M-NaCl revealed that cyclic coiled DNA ($S_{20,w} = 40$ s) is converted by the action of DNase into a species with a sedimentation coefficient ($S_{20,w}$) of 17 s. In some experiments a small amount of material (not exceeding 10%) sedimenting at an intermediate speed ($S_{20,w} = 25$ s) was observed. The fraction of 40 s component as a function of the time of DNase action is plotted in

Fig. 6. The biological activity of the samples is also plotted in this Figure. It will be seen that the decrease in biological activity closely parallels the decrease of 40 s component as time of DNase action proceeds. In the following section it will be shown that the 17 s component formed in these experiments is identical to the extended cyclic form of native double-stranded ϕX174 DNA. The fact that the extended cyclic DNA is approximately one-tenth as active in the biological assay as the coiled form of double-stranded ϕX174 DNA is in agreement with the observed correlation between biological inactivation and change in sedimentation.

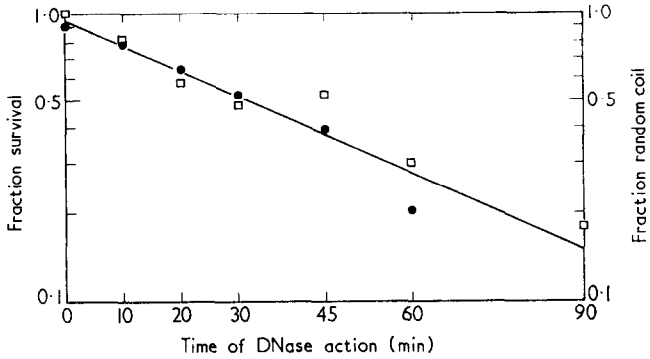


FIG. 6. The biological inactivation (left-hand ordinate) of cyclic coil DNA (□).

Fraction of cyclic coil (right-hand ordinate) *versus* time of DNase action in minutes (●). This fraction was determined as in Fig. 3. DNA concentration: 40 μg/ml. DNase concentration: 0·0002 μg/ml.

The nature of the 25 s component observed in some experiments remains unidentified. An obvious interpretation is that the 25 s component represents an intermediate in the conversion of the cyclic coil (40 s) into the extended cyclic helix (17 s). However, further analysis is required to answer this question.

(d) *Electron microscopy*

Preparations of coiled double-stranded ϕX174 DNA before and after treatment with DNase were subjected to electron microscopy using the Kleinschmidt technique.

Plate III(a) represents an electron micrograph of a preparation which according to ultracentrifugal analysis contained no less than 90% cyclic coil.

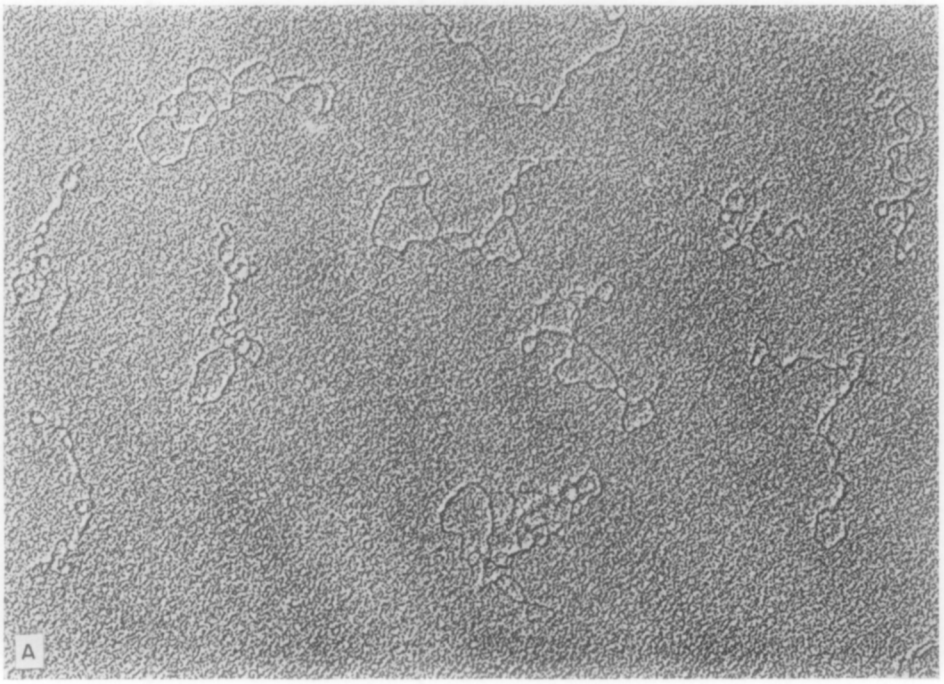
The majority (83%) of the molecules (a total of 3688 were examined) were present as blobs either with or without protruding threads.

The electron micrograph of coiled DNA after treatment with DNase (containing 50% cyclic coil and 50% 17 s component) is presented in Plate III(b). Of these molecules 54% (2136 inspected) were in the form of open or half-open rings and rods.

It is concluded that DNase converts the cyclic coil into the extended circular DNA. Further evidence for this conclusion will be given in the following paper (Pouwels, Knijnenburg, van Rotterdam, Cohen & Jansz, 1968).

4. Discussion

The natural occurrence of circular DNA is not a new concept, but only recently has physical evidence for the circularity of a great number of DNA molecules been obtained. The identification of circular structures was accompanied by the un-



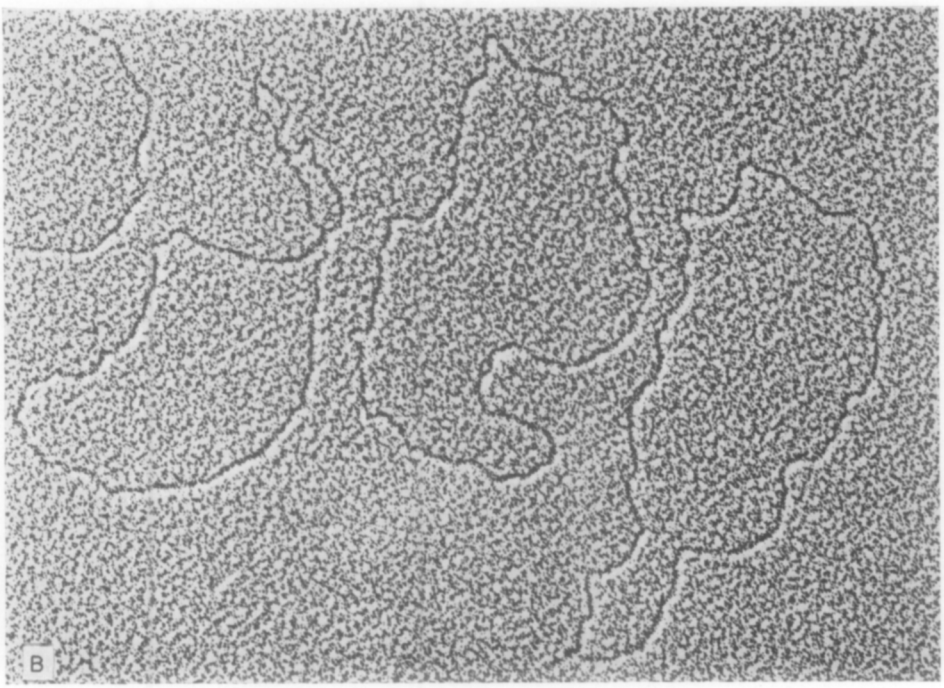


PLATE I. (A) Electron micrograph of twisted cyclic ϕX174 DNA. $\times$62,550.
(B) Electron micrograph of extended cyclic ϕX174 DNA. $\times$100,000.

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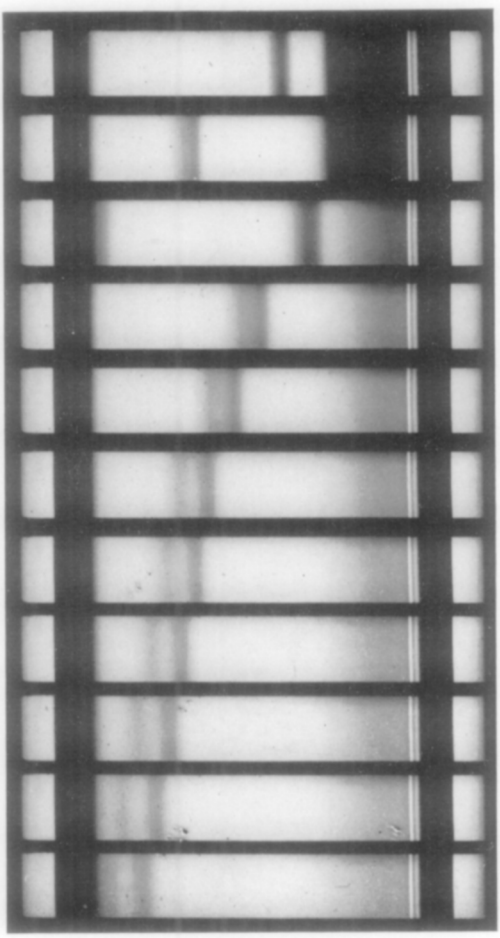
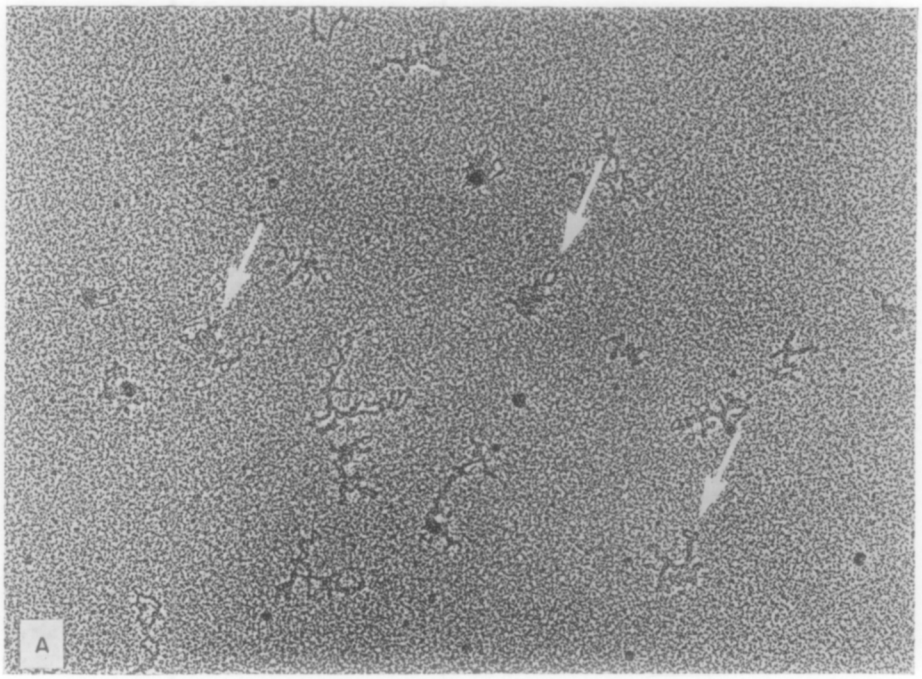


PLATE II. Separation of circular and linear single-stranded ϕX174 DNA.

Analyses were performed in CsCl ($\rho = 1{\cdot}35$) containing 0·01 M-phosphate at pH 12·5 in an AN-D rotor at 47,660 rev/min.



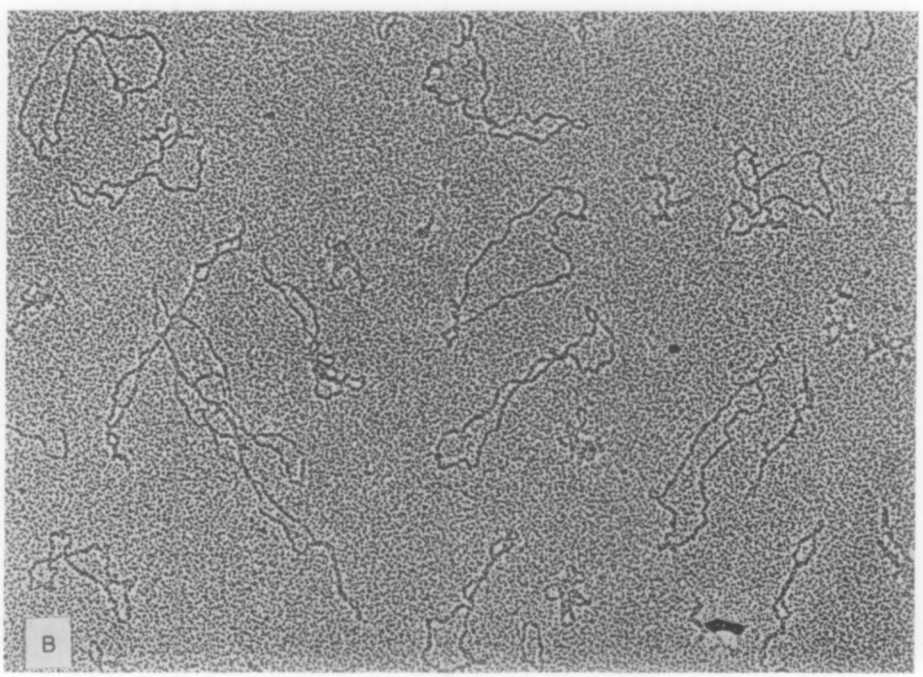


PLATE III. (A) Electron micrograph of cyclic coil (see arrows). ×56,000.
(B) Electron micrograph of cyclic coil after treatment with DNase (30 min). ×56,000.

expected finding that ring-shaped double-stranded DNA contains, superimposed upon the Watson–Crick double helix, tertiary turns which give these structures a twisted configuration.

In the present experiments it has been shown that not more than one single-strand chain break by DNase, which may occur in a random fashion, converts the twisted into the open ring form. The finding that the converting hit has a relatively greater effect on the biological activity than the following single-strand chain breaks is not unexpected. The converting hit induces a conformational change of the molecule which may affect the infectivity for spheroplasts. The further biological inactivation follows an exponential course. The slope of this latter curve differs by a factor of 20 from the slope of the curve which relates the conversion of the twisted into the extended circular form. The most obvious explanation is that only one-twentieth of the single-strand chain breaks in DNA are lethal because of the presence of a mechanism within the host cell which is able to repair a fraction of the chain breaks. An analogous situation exists for the inactivation of double-stranded ϕX174 DNA by ultraviolet light. In this case a fraction of the ultraviolet lesions is repaired within the host cell by an enzymic mechanism, since double-stranded ϕX174 DNA shows a radiosensitivity which is very near that of single-stranded ϕX174 DNA (which is non-reactivable) when assayed in host cells in which reactivation is lacking. The recent discovery of an enzyme which is able to seal single-strand breaks in double-stranded DNA, may be responsible for the repair of single-strand breaks in DNA (Gellert, 1967). In this respect, it would be interesting to test the biological activity of DNase-treated double-stranded DNA using spheroplasts in which the sealing enzyme is lacking.

The finding that DNase converts the cyclic coil by a single or a very few single-strand chain breaks into the native extended ring structure is quite unexpected, and this finding sets certain limits to the structure of the coiled molecule. The observed conversion explains several of the biological properties of the cyclic coil. It seems highly unlikely that the cyclic coil, as such, is able to initiate the infection process. Rather the possibility should be considered that nucleases within the host cell bring about the conversion of the supercoil into the helical form prior to the initiation of phage replication. The higher biological activity of the cyclic coil as compared to double-stranded ϕX174 DNA would then be due to a more efficient penetration of the cyclic coil into spheroplasts.

Similarly, the ultraviolet-irradiated cyclic coil may be converted into the helical extended ring structure prior to or during the process of host-cell reactivation. Host-cell reactivation is known to involve the introduction of single-strand chain breaks at either side of the ultraviolet lesion, which is followed by the excision of the single-strand region and the repair replication of the gap (Boyce & Howard-Flanders, 1964; Setlow & Carrier, 1964). Repair replication requires the presence of the complementary base sequence in the region of excision. This condition is not likely to be fulfilled in the case of the denatured cyclic coil. However, the helical structure may be restored in the preliminary stages of host-cell reactivation, creating a helical structure which is susceptible to repair replication.

Evidence in this and the following paper suggests a model for the cyclic coil which will be discussed (Pouwels *et al.*, 1968).

We thank Professor J. A. Cohen for many interesting discussions and Professor M. Gruber for his interest in this work.

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