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Transfection of *Escherichia coli* Spheroplasts

II. Relative Infectivity of Native, Denatured, and Renatured Lambda, T7, T5, T4, and P22 Bacteriophage DNAs

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The change of infectivity of phage DNAs after heat and alkali denaturation (and renaturation) was measured. T7 phage DNA infectivity increased 4- to 20-fold after denaturation and decreased to the native level after renaturation. Both the heavy and the light single strand of T7 phage DNA were about five times as infective as native T7 DNA. T4 and P22 phage DNA infectivity increased 4- to 20-fold after denaturation and increased another 10- to 20-fold after renaturation. These data, combined with other authors' results on the relative infectivity of various forms of ϕX174 and lambda DNAs give the following consistent pattern of relative infectivity. Covalently closed circular double-stranded DNA, nicked circular double-stranded DNA, and double-stranded DNA with cohesive ends are all equally infective and also most highly infectious for *Escherichia coli* lysozyme-EDTA spheroplasts; linear or circular single-stranded DNAs are about $\frac{1}{5}$ to $\frac{1}{20}$ as infective; double-stranded DNAs are only $\frac{1}{100}$ as infective. Two exceptions to this pattern were noted: lambda phage DNA lost more than 99% of its infectivity after alkaline denaturation; this infectivity could be fully recovered after renaturation. This behavior can be explained by the special role of the cohesive ends of the phage DNA. T5 phage DNA sometimes showed a transient increase in infectivity at temperatures below the completion of the hyperchromic shift; at higher temperatures, the infectivity was completely destroyed. T5 DNA denatured in alkali lost more than 99.9% of its infectivity; upon renaturation, infectivity was sometimes recovered. This behavior is interpreted in terms of the model of T5 phage DNA structure proposed by Bujard (1969). The results of the denaturation and renaturation experiments show higher efficiencies of transfection for the following phage DNAs (free of single-strand breaks): T4 renatured DNA at 10^{-3} instead of 10^{-5} for native DNA; renatured P22 DNA at 3×10^{-7} instead of 3×10^{-9} for native DNA; and denatured T7 DNA at 3×10^{-6} instead of 3×10^{-7} for native DNA.

In a previous paper (4), it was shown that seven native double-stranded phage DNAs efficiently transfected *Escherichia coli* spheroplasts in the presence of protamine sulfate. In this work, we examine the changes in the infectivity of phage DNAs after denaturation and renaturation. The results reveal a consistent pattern of relative infectivities, show higher efficiencies of transfection for some DNAs, and lay the groundwork for investigating the infective properties of intracellular replicative DNA intermediates, heteroduplex DNAs, etc.

MATERIALS AND METHODS

The strains used in this study have been described previously (3, 4). The method for preparing spheroplasts was the procedure described in the following paper (9).

The method for purifying phage (23), extraction of DNA, and sedimentation analysis of DNAs have been described previously (4).

For many experiments, heat denaturation of phage DNAs was performed in 0.01 M Tris-hydrochloride buffer, pH 8.1, containing 1 mM EDTA. As shown in the results, the highly acidic pH of Tris buffer at elevated temperatures induces DNA inactivation (W. F. Studier, personal communication). Thus other heat denaturation experiments were performed in 5 mM sodium phosphate buffer, pH 7.6, containing 1 mM EDTA. After 4 min of heating at the desired temperature, the DNAs were quickly cooled on ice. Undue shear forces during cooling were avoided (because of the shear sensitivity of T4 phage DNA, cooling was often too slow to avoid extensive renaturation). The absorbance of the DNAs was measured at 260 and 280 nm, and the DNAs were then diluted with 0.01 M Tris buffer, pH 8.1, and assayed for infectivity with *E. coli* W3350 spheroplasts.

Alkaline denaturation of phage DNAs was generally performed as described by Davis, Simon, and Davidson (6). Highly purified phage stocks (containing 5 to 100 μg of DNA equivalents per ml) no more than 2 weeks old were used in all experiments. Since T4 and P22 phage plaque formers survived the alkaline treatment, the following modifications were introduced: the NaOH concentration was increased to 0.4 N; the phage was added last without touching the tip of the pipette to the glass tube; and the final dilution of denatured DNA (in 0.01 M Tris buffer, pH 8.1) was sometimes heated for 20 min at 55 C to destroy residual phage plaque formers.

Renaturation of alkali-denatured DNAs was performed in the presence of formamide (Matheson, Coleman, and Bell; reagent grade) as described by Davis et al. (6).

To separate the light (l) and heavy (r) strands of T7 phage DNA, the modified technique (16) of Summers and Szybalski (21) was employed. Only lots of poly U,G which had been tested for efficient strand separation (Schwartz no. 6801; Miles batch no. 3) were ordered from the manufacturer. ^{3}H-thymidine-labeled T7 phage was prepared by the method of Yamamoto et al. (24) and used in all experiments; a control tube of denatured T7 DNA without poly U,G was also centrifuged in CsCl. After collecting 45 fractions from the bottom of the tubes and locating the sharp radioactive peaks of r and l strands as well as the denatured T7 DNA peak in the control tube, fractions were diluted 1,000-fold with 5 mM sodium phosphate buffer, pH 7.6. These diluted fractions were then assayed with *E. coli* W3350 spheroplasts. Parallel samples were heated for 5 min at 95 C, quickly chilled, and also assayed with spheroplasts. This denaturation procedure, which removes poly U,G from the separated strands, increased the infectivity of the l and r strand DNA five- to sevenfold (Table 2). Attempts to remove poly U,G by incubation with 0.4 N NaOH for 4 h at 37 C resulted in a 1,000-fold decrease of infectivity.

TABLE 1. *Transfection after denaturation and renaturation of phage DNAs*[a]

Phage DNA	Number of infective centers for:		
	Native DNA	Denatured DNA	Renatured DNA
T7	168	998	142
T4	20 (6 plates)	119 (6 plates)	2,072 (6 plates)
P22	343 (6 plates)	299 × 10 (6 plates)	5,405 × 20 (6 plates)
Lambda	89	0	132
T5	2,443	10	1,268

[a] A sample of highly purified phage (containing 5–100 μg of DNA equivalents per ml) was extracted twice with phenol, the aqueous phase was diluted at least 100-fold in 0.01 M Tris buffer, pH 8.1, and transfection assays were performed to determine native DNA infectivity. Another sample of phage was added to 0.1 to 0.4 N NaOH in the presence of EDTA (6) to release denatured DNA from the phage; after 10 min at room temperature, a sample was diluted at least 100-fold in 0.01 M Tris buffer, followed by transfection assays to determine denatured DNA infectivity. The denatured DNA was incubated with Tris-hydrochloride and formamide according to Davis et al. (6) for at least 12 h to achieve renaturation. This DNA was diluted at least 100-fold before transfection assays. All transfection assays were performed at concentrations below DNA saturation. Where plaque numbers are multiplied by 10 or 20, this indicates a corrective dilution factor. P22 DNA transfection assays were performed with nonrestricting *E. coli* KF$^+$ $r_K^-m_K^-$ spheroplasts (3). All transfection assays were performed in triplicate, unless otherwise noted.

RESULTS

Figure 1 shows the effect of heat denaturation upon the infectivity of lambda phage DNA. There is a sharp drop in infectivity between 76 and 78 C, where the hyperchromic shift also occurs. Figure 2 shows the results of a similar experiment with T7 phage DNA. Surprisingly, the infectivity of the DNA increases 10-fold during the hyperchromic shift. At higher temperatures, the infectivity declines very slowly. Similar results were obtained with infective P22 and T4 phage DNAs.

If the highly infective forms of heated T7, T4, and P22 DNAs were a product of an intermolecular rearrangement, their formation should be very sensitive to dilution before heat denaturation. Figures 3a and 3b show the results obtained when P22 DNA was denatured at concentrations differing by a factor of ten: the relative increase of infectivity at the hyperchromic shift was nearly the same in both experiments. (As explained in the legend to Fig. 3, the rather rapid decline of infectivity at higher temperatures than 80 C is due to DNA degradation at the low pH of Tris buffer. This artifact has no effect on the results of these experiments since [i] the hyperchromic shift is complete below 80 C, [ii] denaturation curves have been performed in phosphate buffer with the same results below 80 C, and [iii] the alkaline denaturation experiment [Table 1] independently confirm the conclusions from the heat denaturation experiments.) Thus the increased infectivity of the heated DNA is caused by some intramolecular rearrangement.

Another experiment was performed to see if the presence of protamine sulfate in the spheroplast suspension had any effect on the increased infectivity after heating. The result (Fig. 4) shows that the T7 phage DNA infectivity still increased after denaturation even when protamine sulfate was omitted from the spheroplast assays.

Independent confirmation of the heat denaturation data was obtained in alkaline denaturation experiments. Table 1 shows that T7, T4, and P22 DNA infectivity increased 10-fold after

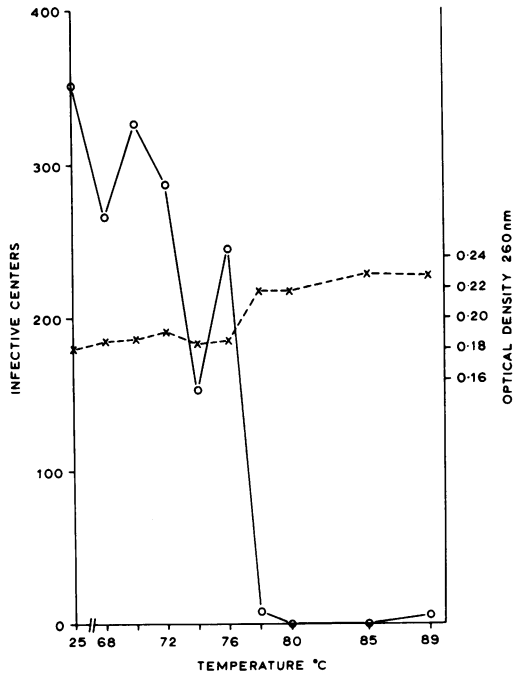

FIG. 1. *Biological melting curve of bacteriophage lambda DNA. Samples (4 ml) of DNA at a concentration of 1.4 × 10^{11} molecules/ml of 0.01 M Tris buffer (pH 8.1), containing 1 mM EDTA, were heated for 4 min at the temperatures indicated on the abscissa and then rapidly chilled on ice. After measuring the absorbance of each sample at 260 and 280 nm, a portion was diluted 10,000-fold in 0.01 M Tris buffer (pH 8.1) and then triplicate assays for infectivity were performed with spheroplasts of E. coli W3350 plus protamine sulfate. The number of infective centers counted on three plates is plotted on the ordinate. The same results were obtained when the DNA was heated in 5 mM phosphate buffer. Symbols: ——, infective centers; -----, absorbance at 260 nm.*

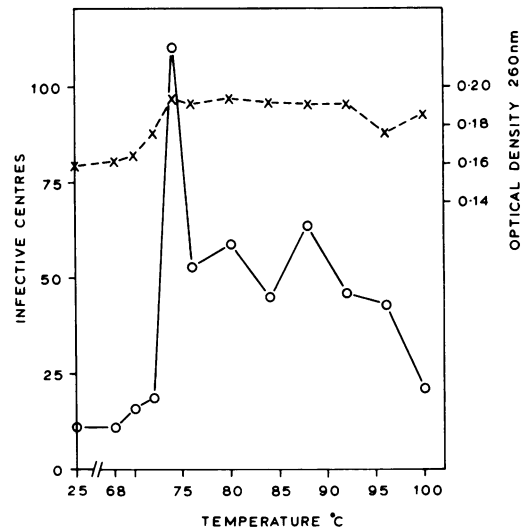

FIG. 2. *Biological melting curve of bacteriophage T7 DNA. Samples of DNA, at a concentration of 1.3 × 10^{11} molecules/ml of 5 mM Na_2HPO_4 (pH 7.8) buffer containing 1 mM EDTA, were treated as indicated in Fig. 1. After 100-fold dilution in Tris buffer, triplicate assays were performed with spheroplasts. Symbols: ——, infective centers; -----, absorbance at 260 nm.*

alkaline denaturation, as expected from the results with heat denaturation.

Convincing evidence that the increased infectivity of T7 DNA after denaturation is due to single-stranded molecules is presented in Table 2. Here the relative infectivity of native, denatured, and the separated l and r strands of T7 phage DNA are compared. Native T7 DNA infectivity is inhibited somewhat by double-stranded calf thymus DNA whereas single-stranded fd DNA has no effect, as expected from previous results (1, 19). Denatured T7 DNA is about 10 times as infective as native DNA (Table 2), and its infectivity is inhibited by single-stranded DNA but not by double-stranded DNA (1, 19), indicating that the infective molecule is single-stranded DNA. Purified T7 l-strand DNA is also 10 times as infective as native DNA, and its infectivity is selectively inhibited by single-stranded DNA; however, this effect is only seen after the l-strand DNA has been denatured again to remove bound poly U, G (Table 2). Before denaturation, l-strand DNA infectivity is about as high as native T7 DNA infectivity and its infectivity is not inhibited by either double-stranded or single-stranded DNA (Table 2). Finally, r-strand DNA infectivity behaves much like l-strand infectivity (Table 2). However, denatured r-strand infectivity is not as high as denatured l-strand infectivity. Since much more poly U, G binds to T7 r-strand DNA than to l-strand DNA (21), we attribute the lower infectivity of denatured r-strand DNA to an incomplete removal of poly U, G.

When T5 phage DNA was subjected to heat denaturation, the infectivity behaved in one of two ways. In Fig. 5a an example is given of a temporary rise in infectivity which occurs at the beginning of the multi-step hyperchromic shift (11) and is followed by a steep decline. In Fig. 5b, an example is given of the steep decline of infectivity with the hyperchromic shift, which is similar to the results observed with lambda DNA (Fig. 1).

Another series of experiments was now performed to check on the heat denaturation data and to assess the infectivity of renatured DNAs (Table 1): five different phage DNAs were

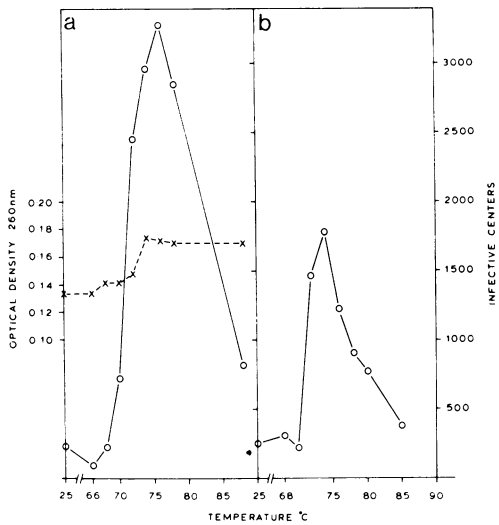

FIG. 3. *a, Biological melting curve of bacteriophage P22 DNA. DNA samples at a concentration of 1.3 × 10^{11} molecules/ml in 0.01 M Tris buffer (pH 8.1), containing 1 mM EDTA, were heated and chilled as described in Fig. 1. Triplicate assays were performed on 10-fold dilutions of DNA using E. coli KF+ 993 $r_K^-m_K^-$ spheroplasts (3) and S. typhimurium SB 1330 $r_{LT}^-m_{LT}^-$ indicator (3). The sharp decrease of infective centers above 80 C is due to the low pH of the Tris buffer at these temperatures. Symbols: ——, infective centers; -----, absorbance at 260 nm. b, The DNA of Fig. 3a was diluted 10-fold in 0.01 M Tris buffer (pH 8.1) and then heated and chilled quickly. Triplicate assays were performed with E. coli KF+ 993 spheroplasts. The sharp decrease of infective centers above 80 C is due to the low pH of the Tris buffer at these temperatures.*

denatured with alkali and then renatured in the presence of formamide. The results fall into three distinct patterns: T7 DNA infectivity increases 10-fold after denaturation and then falls to the level of native DNA infectivity after renaturation. In contrast, T4 and P22 DNA infectivity increase after denaturation and then increase another 10- to 40-fold after renaturation. Both lambda and T5 phage DNAs lose infectivity after denaturation and regain the native level of infectivity after renaturation. Despite the apparent complexity of these patterns, a simple coherent scheme for these infectivity changes has been developed (see Discussion).

DISCUSSION

The effect of both heat and alkaline denaturation on infectivity of several different phage DNAs were similar: T7, T4, and P22 phage DNA infectivity increased about 10-fold, whereas the infectivity of both lambda and T5 phage DNA declined at least 100-fold (Fig. 1-5, Table 1). Hotz and Mauser (10) previously found that T1 phage DNA infectivity increased 40- to 80-fold after both heat and alkaline denaturation, similar to the data for T7 phage DNA in this paper. Veldhuisen and Goldberg (23) also found that T4 DNA transformation activity increased after heat denaturation. Both the isolated heavy and light single strands of T7 DNA are conclusively identified as the infective structures (Table 2). After denaturation, T4 and P22 DNA infectivity rose as much as that of T7 DNA; therefore, we assume that the separate strands of these molecules are also both highly infective.

In the left column of Fig. 6, a simple model to explain how single strands of T7, T4, or P22 phage DNA might lead to an infective center is

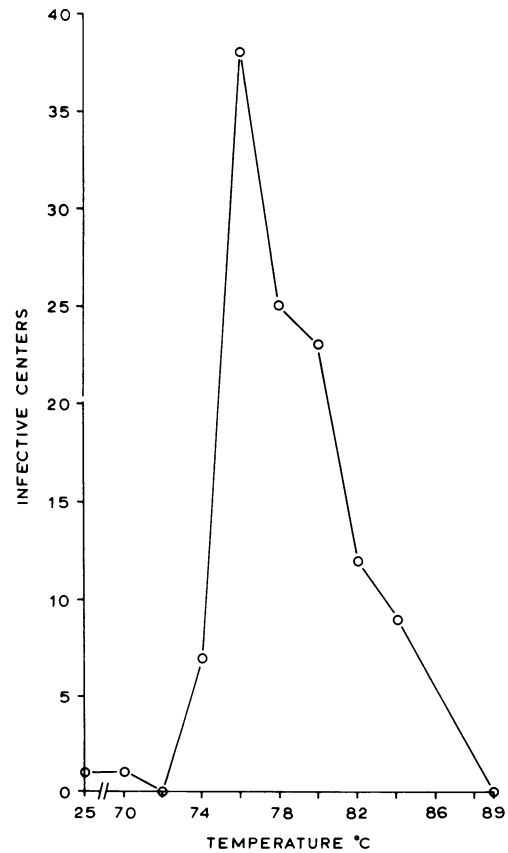

FIG. 4. *Biological melting curve of bacteriophage T7 DNA using spheroplasts without protamine sulfate. DNA samples at a concentration of 1.2 × 10^{10} molecules/ml of Tris buffer (pH 8.1), containing 1 mM EDTA, were heated and then chilled. Triplicate assays were performed on undiluted DNA with spheroplasts of E. coli W3350 without protamine sulfate. The sharp decrease of infective centers above 80 C is due to the low pH of the Tris buffer at these temperatures. Symbol: ——, infective centers.*

proposed: after entry of a single-stranded DNA molecule into a spheroplast, primer-directed replication by the host DNA polymerase regenerates the native phage DNA molecule; this molecule then initiates the infective cycle. The strongest support for this model comes from the results of Table 1: the light strand of T7 phage DNA, which cannot be used as a template for the synthesis of T7 RNA (21) is fully as infective as the heavy strand of T7 phage DNA. The model of Fig. 6 readily explains this result since the complementary DNA strand is made to allow T7 messenger synthesis to proceed.

TABLE 2. *Transfection by native, denatured, and separated T7 DNA strands*

Type of DNA	Average no. of plaques per plate[a]	Average no. of plaques per plate with 0.5 μg of calf thymus DNA added to assays	Average no. of plaques per plate with 0.5 μg of UV-irradiated, single-stranded fd phage DNA added to assays
Native	46	25	60
Denatured DNA from CsCl gradient without poly U, G	310 (331)	323 (321)	60 (71)
T7 l strand	371 (58)	369 (73)	66 (52)
T7 r strand	90 (15)	73 (11)	6 (7)

[a] All assay tubes contained 1.6×10^9 double-stranded DNA equivalents (8.4×10^7 double-stranded molecules gave 1 counts/min). Plaque numbers in parentheses for denatured, l, and r strand DNA indicate values obtained before heat denaturation to remove bound poly U, G.

Lambda phage DNA lost infectivity after both heat and alkaline denaturation (Fig. 1, Table 2). Since cohesive ends of lambda DNA are essential for infection (13), their loss might account for this result. In the second column of Fig. 6, the same host-catalyzed DNA synthesis of the complementary strand which is invoked for T7, T4, and P22 DNAs above is shown to lead to the masking of one cohesive end while the other cohesive end is lost (since it is located on the other single strand). The lack of infectivity of single-stranded lambda phage DNA in the helper assay (7) also supports this model. Although the loss of infectivity of single-stranded lambda DNA in our assay precludes a study of its biological properties, one great advantage of this system is that it permits the study of the infective properties of lambda heteroduplex molecules without interference from nonhybridized, contaminating single-stranded DNA.

The complex behavior of T5 phage DNA infectivity after heat denaturation is more puzzling. One explanation for the transient rise of infectivity sometimes seen before the hyperchromic shift (Fig. 5a) is that a small piece of

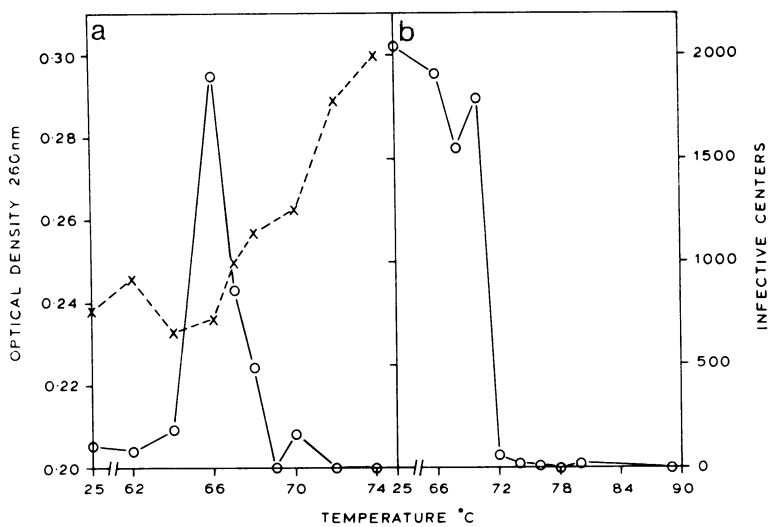

FIG. 5. *a, Biological melting curve of bacteriophage T5 DNA. DNA samples at a concentration of 7.3×10^{10} molecules/ml in 0.01 M Tris buffer (pH 8.1), containing 1 mM EDTA, were heated at various temperatures and then chilled quickly. After measuring the absorbance at 260 nm, samples were diluted 200-fold in 0.01 M Tris buffer and assayed with spheroplasts of E. coli W3350 in the presence of protamine sulfate. Similar results were obtained when the DNA samples were heated in 5 mM phosphate buffer. Symbols: ——, infective centers found on three plates; -----, absorbance at 260 nm. b, Another biological melting curve of bacteriophage T5 DNA. DNA samples at a concentration of 1.3×10^9 molecules/ml were treated as described in the legend to Fig. 5a. Undiluted DNA was assayed with spheroplasts. Symbol: ——, infective centers found on three plates.*

TABLE 3. *Maximum infectivity of various forms of DNA for lysozyme-EDTA spheroplasts in the presence of protamine sulfate*[a]

DNA	Structure of DNA					
	Covalent, double-stranded circles	Nicked, double-stranded circles	Double-stranded DNA with cohesive ends	Single-stranded circles	Linear, single-stranded DNA	Linear, double-stranded DNA
ϕX174	5×10^{-2} (4, 19)	5×10^{-2} (4, 19)		10^{-2} (4)	Not infective (8)	
Lambda	2×10^{-4} (15)	10^{-3}[b] (15)	10^{-3}[b] (4, 15)	3×10^{-6}[c] (14)	Not infective (7)	Not infective (20)
T7					1.5×10^{-6}	3×10^{-7}
T4			10^{-3} (renatured)		5×10^{-5} (denatured)	10^{-5}
P22			3×10^{-7} (renatured)		1.5×10^{-8} (denatured)	3×10^{-9}

[a] Infectivity is defined as the number of infective centers obtained from one molecule of DNA. In the parentheses pertinent references are listed.

[b] These data are supported by the results of Enquist, Skalka, and Benzinger (manuscript in preparation).

[c] Data of Kiger, Young, and Sinsheimer (14). We assume that the infectivity of lambda single-stranded circular DNA is not enhanced by protamine sulfate in the spheroplast assay system, although we have not tested this assumption. Both single-stranded ϕX174 and fd phage DNA infectivity is not enhanced by protamine sulfate (4).

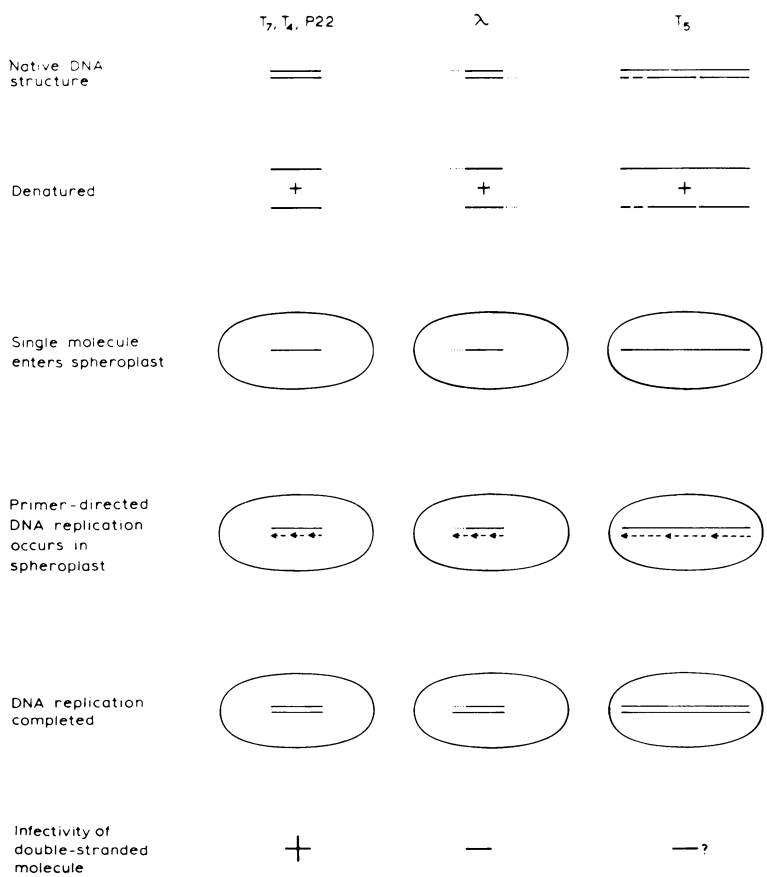

FIG. 6. *Models for: the infectivity of single-stranded T4, P22, and T7 DNAs (column 1); for the loss of infectivity of lambda (column 2) and T5 phage DNA (column 3) after denaturation. The structure of mature T5 phage DNA is depicted according to the model of Bujard (5).*

DNA on the multiply nicked strand is lost at temperatures just below the melting point (5, 11); such a partially single-stranded DNA molecule may be more infective than native T5 phage DNA.

The loss of infectivity of T5 phage DNA at higher temperatures and in alkali (Table 2) may be explained by reference to Fig. 6. A molecule of single-stranded DNA entering a spheroplast is converted into a native double helix by host DNA polymerases; this molecule is devoid of single-strand interruptions. If the nicks are essential for infectivity, such a molecule should not be infective. A direct test of this hypothesis is to measure the effect of polynucleotide ligase, which repairs all of the nicks of T5 phage DNA (12), on the infectivity of T5 phage DNA.

The results of the DNA renaturation experiments (Table 1) remain to be interpreted: T7 and lambda DNA infectivity returned to the level of native phage DNA after renaturation, suggesting that the original structure was regenerated. T5 phage DNA infectivity was also recovered after renaturation (Table 1). However, this result is difficult to interpret because of the many different DNA structures which could be generated from the fragments produced after denaturation and because of the large terminal redundancy of the DNA (18).

Surprisingly, renaturation of T4 and P22 phage DNA resulted in a further 10- to 20-fold increase of infectivity of these molecules. Perhaps a structural difference between T7 and these two phage DNAs could account for their different infectivity after renaturation. It is known that T7 phage DNA has a unique DNA sequence while both T4 (Thomas and MacHattie, 1964) and P22 (17) have circularly permuted DNA. Thus, renaturation of T7 DNA will regenerate the original native structure whereas P22 and T4 DNAs will renature to form double-stranded structures with cohesive ends or circles. Perhaps such molecules are more highly infective than even single-stranded T4 or P22 phage DNAs; this hypothesis is supported by the data of Table 3 which combines the data obtained in this paper with the maximum efficiency of transfection observed for different forms of lambda and ϕX174 DNAs. Several consistent features emerge from this tabulation: qualitatively, circular double-stranded DNA and DNA with cohesive ends are more infective than single-stranded DNAs, which in turn are more infective than linear, double-stranded DNAs. Quantitatively, the high and equivalent efficiency for covalent and nicked double-stranded circles (ϕX179, lambda) and for double-stranded DNA with cohesive ends (lambda) is striking. Renatured T4 DNA (which is either circular or has cohesive ends) is just as efficient (10^{-3}) as lambda DNAs. With the exception of single-stranded circular lambda DNA, all single-stranded forms are $\frac{1}{5}$ to $\frac{1}{20}$ as infective as the double-stranded circles or DNA with cohesive ends. Linear, native, double-stranded DNAs are about $\frac{1}{10}$ as infective as the single-stranded DNAs. This pattern might be very useful in predicting the infectivity of various forms of intracellular replicating DNA intermediates which have not been tested in transfection assays (such as T4, T7, P22).

The mechanism underlying the differential infectivity of the different DNA structures compared in Table 3 is still obscure. Either hypothesis discussed in a previous paper (4), differential DNA uptake, or differential sensitivity to intracellular nucleases could explain the results.

Nevertheless, a substantial increase of maximum transfection efficiency has been found for several intact phage DNAs: using renatured T4 phage DNA increases the efficiency to 10^{-3} infective centers per DNA molecule compared to the previously reported value (4) of 10^{-5} for native DNA; using renatured P22 DNA increases the efficiency from 3×10^{-9} to 3×10^{-7}; and using denatured T7 DNA increases the efficiency from 3×10^{-7} to 3×10^{-6}. Where the structure of the transfecting DNA is not important, these increases in transfection efficiency should be quite helpful.

ACKNOWLEDGMENTS

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