Transfection of *Escherichia coli* Spheroplasts

I. General Facilitation of Double-Stranded Deoxyribonucleic Acid Infectivity by Protamine Sulfate

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The addition of 25 μg of protamine sulfate per ml to lysozyme-ethylenediaminetetraacetic acid spheroplasts of *Escherichia coli* stimulates transfection not only for T1 phage deoxyribonucleic acid (DNA; Hotz and Mauser, 1969) but also for the following phage DNA species: lambda, 10,000-fold to an efficiency of 10<sup>-3</sup> infective centers per DNA molecule; φX174 replicative form, 300-fold to an efficiency of 5 x 10<sup>-5</sup>; fd replicative form, 300-fold to 10<sup>-4</sup>; T7, 300-fold to 3 x 10<sup>-3</sup>. Three native phage DNA species were not infective at all in the absence of protamine sulfate but were infective in the presence of protamine sulfate with the following efficiencies:

- T4, 10<sup>-2</sup>
- T5, 3 x 10<sup>-4</sup>
- P22, 3 x 10<sup>-3</sup>

The effect of protamine sulfate is specific for double-stranded DNA. The application of infectivity assays to the study of phage DNA replication, recombination, prophage integration, prophage excision, and interspecies transfection are discussed.

MATERIALS AND METHODS

**Strains.** Phages T<sup>+</sup> and T<sup>−</sup> and their host *Escherichia coli* B/1 were the gift of W. Sauerbier; P22 H5 phage and the indicator *Salmonella typhimurium* LT-7 SB 1330 *str leu 119* fer were donated by P. E. Hartman; T4D and T4oB phage and the indicator strain *E. coli* B were gifts of E. Kutter and B. Alberts; R17 phage and the indicator strain *E. coli* D10 meth<sup>−</sup> ribonucleas<sup>−</sup> were obtained from R. F. Gesteland; a clear mutant of lambda phage as well as φX174 and fd phage and the corresponding indicator strains *E. coli* C600, E. coli C, and E. coli K12 F<sup>−</sup> meth<sup>−</sup> rec<sup>−</sup> muc<sup>−</sup> were from our own collection. The spheroplast recipient strains *E. coli* W 3350 gal<sup>−</sup> gal<sup>−</sup> lac<sup>−</sup>, Hfr C64 meth<sup>−</sup> B<sup>+</sup> endol<sup>−</sup>, and K37 F<sup>+</sup> sup<sup>+</sup> were the respective gifts of H. Drexler, H. Hoffmann-Berlinger, and D. Pratt.

**Other materials.** One liter of slant agar contained 15 g of nutrient broth (Difco), 5 g of NaCl, 20 g of agar (Difco), 10 μg of required amino acids per ml, and 5 ml of 1 N NaOH added after sterilization. The following chemicals were purchased: protamine sulfate, U.S.P. injection grade (containing 0.25% phenol as a preservative) from Eli Lilly & Co.; a 30%; solution of bovine “Povite” albumin from Serum Biotic Institute, Frankfurt, Main; lysozyme, Pronase, and ribonuclease from Calbiochem; and electrophoretically purified deoxyribonuclease from Worthington Biochemical Corp.

**Methods Used.** Bacteriophage were purified from crude lysates by polyethylene glycol precipitation followed by CsCl density gradient centrifugation (20); the following phage titers (plate-forming units/milliliter) were found for an absorbance of 1 at 260 nm
- P22 H5, 8 x 10<sup>11</sup>
- T7, 6 x 10<sup>11</sup>
- T5, 1.2 x 10<sup>11</sup>
- T4, 10<sup>11</sup>
- fd, 9 x 10<sup>11</sup>
- φX174, 3.5 x 10<sup>11</sup>
- lambda, 5 x 10<sup>11</sup>
- R17, 1.2 x 10<sup>10</sup>

Nucleic acid was extracted from these phage (suspended in an alkaline buffer containing 1 mM EDTA) by gently rolling with an equal volume of distilled and then water-saturated phenol, collecting the aqueous phase after centrifugation, and repeating this phenol extraction two more times; the final aqueous phase was dialyzed against 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.5 (containing 1 mM EDTA). The ab-
sorbance of the nucleic acids was 1.8- to 2-fold higher at 260 nm than at 280 nm. Where the number of virus particles per milliliter could be calculated from a known extinction coefficient, the number of isolated DNA molecules per milliliter closely corresponded. The double-stranded replicative form DNA species of \( \Phi X174 \) and fd phage were prepared essentially by the technique of Jaenisch et al. (8). The number of DNA molecules per milliliter for each preparation was calculated from the known molecular weights and the relationships 50 \( \mu g/ml = 1 \text{OD/ml at} \ 260 \text{ nm for double-stranded DNA and} \ 33 \ \mu g/ml = 1 \text{OD/ml at} \ 260 \text{ nm for single-stranded DNA.}

The integrity of all of the DNA species was measured by both neutral and alkaline band sedimentation by the method of Studier (17). All of the double-stranded DNA species sedimented homogeneously with the expected S values in neutral CsCl; thus, few if any double-stranded breaks had occurred in these DNA species. Both fd and \( \Phi X174 \) phage DNA sedimented in neutral CsCl as expected for circular DNA species. In alkaline CsCl, more than 50% of the DNA sedimented with the velocity expected for whole single strands in the case of lambda, T4, T7, and P22 DNA. T5 phage DNA showed extensive nicking in alkaline CsCl runs. Both fd and \( \Phi X174 \) replicative forms and single-stranded phage DNA species sedimented homogeneously at rates expected for covalently linked circles.

Method for preparing spheroplasts. This technique is a slight modification of the procedure of Guthrie and Sinsheimer (5). Not all of the steps presented here have been checked to see whether they are essential for obtaining competent cells.

E. coli W3530 (most competent strain), E. coli Hfr C64 (competence generally 1 to 10% of W3530), or E. coli K37 (competence generally 10 to 30% of W3530) were transferred from a 2-month-old slant to a fresh slant and incubated at 25 C for 56 hr. An overnight culture of bacteria in Fras and Jerrel's (4) glycerol medium was prepared. The next morning, 1 ml of bacteria was added to 200 ml of fresh medium, and the culture was shaken until the cell count reached 5 \( \times \) 10\(^8\) bacteria/ml (an optical density of 1 at 550 nm in a model 2400 Gilford spectrophotometer). The bacteria were centrifuged at room temperature and resuspended in 3.5 ml of 1.5 M sucrose, followed by 1 ml of 30% Povite albumin, and 0.2 ml of lysozyme (2 mg/ml in 0.25 M Tris buffer, \( \text{pH} 8.1 \)). Then 0.4 ml of unbuffered 4% EDTA was added, and 95 ml of PA medium (5) was added 40 to 120 sec later. After 10 min at room temperature without stirring, 2 ml of 10% MgSO\(_4\) and 0.25 ml of 1% protamine sulfate were added. The suspension was placed on ice and tested with \( \Phi X174 \) phage and replicative form DNA for competence 0.5 hr after preparation (5). Four hours later, the results of these transfection assays were evaluated; if the efficiency was high, these spheroplasts were used for transfection with other DNA species. Spheroplasts were usually at peak competence levels between 4 and 10 hr after preparation and then decayed rapidly; for the small fd and \( \Phi X174 \) DNA species, competence was often stable for as long as 3 weeks.

The assay for larger DNA species (lambda, T7, T5, P22, and T4) was modified from that of Guthrie and Sinsheimer (5) in the following manner. DNA was diluted (by using sawed-off 1-ml plastic pipettes for T5 and T4 DNA) in 0.01 M Tris buffer (\( \text{pH} \ 8.1 \)), instead of the usual 0.05 M Tris buffer; ionic strengths higher than 0.02 in the assay inhibit transfection by the larger DNA species as has previously been noted by Meyer et al. (11). An equal volume of spheroplasts was added to the DNA at 30 C, and incubation was continued for 8 to 10 min. The infected cells were then mixed with 3 ml of PAM soft agar (reference 5; containing 1% Povite albumin and phage indicator bacteria) and poured on the appropriate bottom-layer agar plates used in phage assays (\( \text{pH} \) of these plates always adjusted to between 7 and 8). The plates were incubated at 37 C and scored for plaques. All experiments were accompanied by controls showing the absence of infective phage from the spheroplasts, media, and phage DNA species. The presence of mature phage 8 to 10 min after spheroplast addition was ruled out by parallel assays treated with chloroform; for lambda, fd, and \( \Phi X174 \) DNA species, phage-resistant spheroplasts were used without affecting the kinetics or yield of infection. The identity of the infective nucleic acids was established by their sensitivity to electrophoretically purified deoxyribonuclease and to heat denaturation above the known Tm and by their resistance to ribonuclease and Pronase. An amber and a temperature-sensitive T4 phage mutant DNA gave the expected ratio of infective centers under permissive and nonpermissive conditions. In addition, both infective T4 and T5 phage DNA species were sensitive to mild shear on a vortex mixer, whereas the other DNA species were resistant; in fact, T7 DNA transfection was increased 2 to 3 times by prior vortex treatment.

The efficiency of transfection is defined here as the number of infective centers obtained for one molecule of DNA added to the assay tube.

RESULTS

Table 1 shows the stimulatory effect of protamine sulfate for transfection by seven different double-stranded DNA species. \( \Phi X174 \) and fd replicative-form transfection as well as T7 DNA transfection were stimulated 300-fold, whereas lambda DNA transfection was stimulated 10,000-fold. In the absence of protamine sulfate, T4, T5, and P22 DNA species were not detectably infective, whereas in the presence of protamine sulfate, efficiencies of transfection of \( 10^{-5} \), \( 3 \times 10^{-4} \), and \( 3 \times 10^{-3} \), respectively, were obtained. However, both of the single-stranded phage DNAs' and one phage RNA's (one experiment) transfection were not stimulated by protamine sulfate. In fact, an transient inhibition of \( \Phi X174 \) phage DNA transfection was observed with two-thirds of all fresh spheroplast preparations (data not shown).

Figure 1 shows that the number of infective centers was directly proportional to the number of lambda DNA molecules in the assay tube over
TABLE 1. Transfection of W3350 spheroplasts with and without protamine sulfate

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Spheroplasts without protamine sulfate:</th>
<th>Spheroplasts with 25 μg of protamine sulfate added per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum observed* efficiency of transfection</td>
<td>Maximum observed* efficiency of transfection</td>
</tr>
<tr>
<td>Double-stranded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lambda phage</td>
<td>10^2 (16)</td>
<td>10^2 (26)</td>
</tr>
<tr>
<td>φX174 replicative form</td>
<td>1.7 × 10^-4 (75)</td>
<td>5 × 10^-2 (75)</td>
</tr>
<tr>
<td>fd Replicative form</td>
<td>3 × 10^-8 (5)</td>
<td>10^-6 (7)</td>
</tr>
<tr>
<td>T7 phage</td>
<td>10^-4 (25)</td>
<td>3 × 10^-7 (37)</td>
</tr>
<tr>
<td>T4 phage</td>
<td>ND (10)c</td>
<td>10^-4 (14)</td>
</tr>
<tr>
<td>T5 phage</td>
<td>ND (5)</td>
<td>3 × 10^-4 (19)</td>
</tr>
<tr>
<td>P22 phage</td>
<td>ND (10)</td>
<td>3 × 10^-4 (22)</td>
</tr>
<tr>
<td>Single-stranded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>φX174 phage</td>
<td>10^-3 (75)</td>
<td>10^-2 (75)</td>
</tr>
<tr>
<td>fd Phage</td>
<td>10^-8 (8)</td>
<td>10^-4 (8)</td>
</tr>
<tr>
<td>R17 phage</td>
<td>~10^-7 (1)</td>
<td>~10^-7 (1)</td>
</tr>
</tbody>
</table>

* Efficiency of transfection is defined as the number of infective centers obtained from one DNA molecule. Only 10% of all spheroplast preparations gave the values listed here. Although the efficiency of transfection in the absence of protamine sulfate varied from preparation to preparation by as much as 100-fold, the relative facilitation of transfection by protamine sulfate was constant. The titer of spheroplasts in all experiments was 5 × 10^8/ml. The numbers in parentheses indicate the number of experiments performed.

* Spheroplasts for these experiments were less efficient than listed in the column “maximum observed efficiency of transfection.”

* Not detectable.

a wide range of concentrations (all other DNA species except T5 phage DNA also showed such a linear dependence). Up to 4 × 10^6 infective centers/ml were obtained at a saturating concentration of about four DNA molecules per spheroplast. At higher DNA concentrations, inhibition of transfection was observed; similar results were seen for T7 and T4 DNA, in agreement with Hotz and Mauser’s (6) data on T1 phage DNA transfection.

**DISCUSSION**

Several plausible hypotheses for the enhancement of transfection by protamine sulfate might be advanced. (i) It facilitates the transport of DNA through the cell wall and cell membrane. This is unlikely because addition of protamine sulfate to the transfecting DNA instead of to the spheroplasts completely inhibits transfection. (ii) It inhibits nucleases which degrade much of the transfecting DNA before it can be expressed. Indeed, some nucleases are precipitated by protamine sulfate in the presence of the cellular nucleic acids [see, for example, Sadowski and Hurwitz (14)]. However, it is not clear how the inhibition of nucleases could explain the specificity of transfection enhancement for double-stranded DNA (see Table 1). Since both linear and circular double-stranded DNA transfection are enhanced
by protamine sulfate and circular single-stranded DNA transfection is not affected, specific inhibition by protamine sulfate of an endonuclease which attacks only double-stranded DNA might be postulated. Endonuclease I of E. coli has such specificity (9) except that it will attack single-stranded DNA at low rates. However, the use of endonuclease I− mutants or the addition of RNA [which strongly inhibits endonuclease I (9)] in transfection assays increases transfection by no more than threefold (21), not enough to account for the 300-fold stimulation of transfection by protamine sulfate. Thus protamine sulfate could only inhibit some as yet undiscovered nuclease or combination of nucleases. (iii) It complexes double-stranded host DNA released from lysed spheroplasts (7); this complex, unlike the free DNA, no longer inhibits the uptake of double-stranded transfecting DNA. This seems the most plausible explanation for the effect of protamine sulfate since the specificity of transfection enhancement for double-stranded DNA can readily be explained. If single-stranded DNA enters the cell by a different mechanism than double-stranded DNA, it may be insensitive to inhibition of uptake by double-stranded DNA released from lysed spheroplasts. Indeed, both Postel and Goodgal (13) and Chilton and Hall (3) have found that the uptake of single-stranded transforming DNA takes place under special conditions where hardly any double-stranded DNA uptake is observed. Furthermore, Sinsheimer (15) has found that transfection by single-stranded DNA is not affected by high concentrations of double-stranded DNA, whereas much lower concentrations of single-stranded DNA do inhibit transfection. A number of other observations can also be explained by the hypothesis. (i) As Hotz and Mauser (6) have observed (see Fig. 1), there is a peak in the DNA saturation curve followed by a decline of infective centers instead of the usual plateau observed in the absence of protamine sulfate. If high concentrations of transfecting DNA competed strongly with inhibitory DNA released from lysed spheroplasts for the binding of protamine sulfate, this might explain the results. (ii) Treatment of spheroplasts in the absence of protamine sulfate with pancreatic deoxyribonuclease will often stimulate transfection by phage RNA (1) two- to fivefold. Perhaps the degradation of inhibitory DNA from lysed spheroplasts enhances the uptake of RNA. Parachyc3 (12) has also shown a weak stimulation of phage RNA transfection by protamine sulfate. (iii) A transient inhibition of single-stranded DNA transfection by protamine sulfate is observed for two-thirds of all fresh spheroplast preparations (data not shown); perhaps this is due to the incomplete lysis of fresh spheroplasts so that excess protamine sulfate is now available to inactivate transfecting DNA. Indeed, a progressive lysis of spheroplasts during storage has been observed (1). (iv) Native double-stranded φX174 replicative form DNA is only slightly infective for penicillin spheroplasts but becomes highly infective after heat denaturation (2); similarly, T4 DNA infectivity increases for penicillin spheroplasts after heat denaturation (19). Penicillin spheroplast populations contain many lysed cells (1). To distinguish among the possibilities mentioned above and others, experiments using labeled transfecting DNA, protamine sulfate, and spheroplasts are now in progress.

Replicative intermediates have been isolated from many different kinds of phage-infected cells and have been characterized by physical chemical techniques; the high efficiencies of transfection presented here and the direct dependence on DNA concentration make the assay ideal for studying the biological properties of such intermediates. A more important application may be to the study of both in vivo and in vitro recombination. If the total complement of intracellular phage DNA can be assayed for recombinants at various times after infection, such mysterious variables as the number of rounds of mating might be determined; furthermore, presumed intermediates in recombination such as the T4 joint molecules described by Tomizawa and Anakura (18) might be tested to see whether they yield recombinants in transfection assays.

The observation that P22 phage DNA (isolated from phage grown on Salmonella typhimurium) can infect E. coli spheroplasts and yield progeny phage (Benzinger and Kleber, submitted for publication) makes it possible to study transfection between different genuses and to determine the limiting factors (such as restriction) in such assays. The assay may also be used in other systems (10).

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LITERATURE CITED


metting curves for the "replicative form" of \( \phi X \) 174 DNA.
Z. Vererbungsl. 94:316-321.
3. Chilton, M., and B. D. Hall. 1968. Transforming activity in
single-stranded DNA from \( B. subtilis \). J. Mol. Biol. 34:439-
451.
4. Fraser, D., and E. A. Jerrel. 1953. The amino acid composition
5. Guthrie, G. D., and R. L. Sinsheimer. 1963. Infection of
bacterial protoplasts with the DNA of bacteriophage \( \phi X \)
6. Hotz, G., and R. Mauser. 1969. Infectious DNA from colo-
phage Tl. I. Some properties of the spheroplast assay
infektioese Substrukturen aus Bakteriophagen VIII. On
the tertiary structure and biological properties of \( \phi X \)
8. Jaenisch, R., Hofscheider, P. H., and A. Preuss. 1969. Isola-
tion of circular DNA by zonal centrifugation: separation of
normal length, double length, and catenated M13 replicative
form DNA and of host-specific "episomal" DNA. Bio-
J. N. Davidson and W. E. Cohn (ed.), Procedures in
York.
Origin of DNA and protein in lambda DNA disrupted cell
Infectious deoxyribonucleic acid from lambda bacterio-
12. Paranchych, W. 1963. Assay of infectious RNA from bacte-
riophage R17. Biochem. Biophys. Res. Commun. 11:28-
33.
stranded" DNA in \( H. influenzae \) and its ability
of deoxyribonucleic acid. I. Purification and properties of
endonuclease II from T4 phage-infected \( E. coli \). Bio-
York.
plaque-forming ability of poliovirus ribonucleic acid with
17. Studier, W. F. 1965. Sedimentation studies of the size and
18. Tomizawa, J., and N. Anraku. 1964. Molecular mechanism of
genetic recombination in bacteriophage \( H \). Joining of
formation of bacteriophage T4, p. 858-863. In L. Grossman
20. Yamamoto, K., B. Alberts, R. Benzinger, L. Lawhorne, and
G. Treiber. 1970. Rapid bacteriophage sedimentation in the
presence of polyethylene glycol and its application to large-
bacteriophage lambda DNA I. Infectivity in a spheroplast