Transfection of *Escherichia coli* Spheroplasts

III. Facilitation of Transfection and Stabilization of Spheroplasts by Different Basic Polymers

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The only compound which fully replaced protamine sulfate in facilitating transfection of *Escherichia coli* spheroplasts by phage DNAs was spermine; poly-L-lysine, poly-L-arginine, DEAE-dextran, histones, and many other polyamines were only slightly effective. Higher-molecular-weight compounds were effective at lower concentrations, and each compound had a sharp concentration optimum. The specificity of the facilitation of transfection is discussed in light of Leonard and Cole's (1972) isolation of a polyamine- or protamine-like, natural competence factor from *Streptococci*. By standardizing growth conditions for spheroplast cultures, storing spheroplasts in minimal medium, and adding both protamine sulfate and polyamines to spheroplasts, reproducible competence levels were obtained. Thus, 95% of all spheroplast preparations gave efficiencies of transfection between 10^{-3} and 3×10^{-4} for lambda DNA; between 10^{-6} and 3×10^{-8} for T7 DNA; and between 3×10^{-6} and 10^{-7} for T5 phage DNA. The stability of the spheroplasts was extended from 10 h to between 2 and 5 days, depending on the DNA used for transfection.

In a previous paper (5), protamine sulfate was shown to facilitate transfection with seven different double-stranded phage DNAs by at least 300-fold. A major difficulty with this and other published *Escherichia coli* transfection systems is that high competence levels are very unstable and that only 1 in 10 spheroplast preparations gives high levels of competence (5).

Other basic polymers were now tested for their effects on transfection. When polyamines as well as protamine sulfate were added to spheroplasts, stable and highly competent preparations were reproducibly obtained.

MATERIALS AND METHODS

The strains used in this study have been previously described (5). Polymers were purchased from the following sources: heparin, calf thymus histones, arginine-rich histones, lysine-rich histones, and poly-L-lysine (mol wt 3,000-5,000) from Nutritional Biochemicals Corp.; spermine tetrahydrochloride, spermidine trihydrochloride, putrescine dihydrochloride, and cadaverine dihydrochloride from Calbiochem. 3,3'-diaminopropylamine; *N,N'*-diethyl-1,4-pentanediamine; 4-dodecyl-diethylene triamine; tetraethylenepentamine; *N,N'*-bis (2-aminoethyl) 1,3-propanediamine; *N,N'*-bis (3-aminopropyl)-1,3-propanediamine; 1,1,4,7,7-pentamethyldiethylene-triamine; and *N,N'*-bis [(3-(2-aminoethylamine) propyl)] ethylene diamine from Eastman Ko-

dak. DEAE-dextran was purchased from Pharmacia, Inc.; poly-L-ornithine HBr (manufacturer's mol wt 76,000) was from Mann Research Laboratories; and poly-L-lysine HCl (mol wt 16,400; 75,000; and 200,000) and poly-L-arginine HCl (mol wt 10,000-20,000) were from Miles Laboratories.

The purification of bacteriophage and extraction of phage DNAs has been described previously (5). One unit of absorbance at 260 nm was assumed to represent 40 μ g of double-stranded DNA. Standard growth medium for bacterial cultures was the following modification of the formula of Fraser and Jerrel (7): 4.5 g of KH_2PO_4 , 8.3 g of Na_2HPO_4 , 15 g of Casamino Acids (Difco), 1 g of NH_4Cl , 10 ml of glycerol, 2.5 ml of 10% MgSO_4 , and 0.3 ml of 0.1 M CaCl_2 , per liter.

The optimal method developed for preparing spheroplasts was altered from the previous procedure (5) in the following manner: the absorbance of the overnight culture was between 8 and 12 in the Gilford spectrophotometer (at 550 nm, bacteria diluted 10-fold before measurement) when 2 ml was transferred to 400 ml of fresh medium. The culture was shaken vigorously at 37°C. When the absorbance of the culture reached 0.6, the cells were centrifuged at room temperature and resuspended in: 2.1 ml of 1.5 M sucrose, followed by 0.6 ml of 30% Povite albumin, 0.12 ml of freshly dissolved lysozyme (2 mg/ml in 0.25 M Tris buffer, pH 8.1), and 0.24 ml of unbuffered EDTA.

After 2 min, 55 ml of minimal PA medium (100 g of sucrose, 1 g of glucose, and 0.5 g of Casamino Acids [Difco] per liter of water) was added. After 12 min at

room temperature without stirring, 1.2 ml of MgSO_4 , and 0.15 ml of 1% protamine sulfate, as well as 0.1 ml of a fresh solution of spermine tetrahydrochloride (250 mg/ml in sterile water), were added. Other basic polymers were also added at this time; if they were added to spheroplasts at later times, their effect on competence was always weaker (no more than 10% of the control). The spheroplasts were stored on ice and assayed for competence with ϕX174 DNAs 3 h later, since full competence was not developed till that time (5). Such spheroplasts worked best with λ and ϕX174 DNAs. For T7 DNA assays, protamine sulfate was often omitted to obtain higher competence levels. For T5 assays, spermidine trihydrochloride (250 $\mu\text{g}/\text{ml}$) or N,N' -bis (3 aminopropyl)-1,3-propanediamine (180 $\mu\text{g}/\text{ml}$) was sometimes substituted for spermine tetrahydrochloride. For λ and ϕX174 DNA assays, spheroplasts were stable for 3 to 5 days. For T5 assays, they were stable for no longer than 3 days. For T7 DNA assays without protamine sulfate, spheroplasts were competent between 24 and 72 h after preparation.

For the purposes of this paper, facilitation of transfection is defined as an increase in transfection efficiency at any time after the preparation of the spheroplasts. Stabilization of transfection is defined as the maintenance of a high level of competence any time beyond the first 10 h.

RESULTS

Table 1 presents the results obtained when a variety of basic polymers was used to facilitate transfection by ϕX174 replicative form DNA. None of the compounds was more than 10% as efficient as protamine sulfate in facilitating transfection, not even the chemically closely related poly-L-arginine. The optimum concen-

tration of the polymers depended inversely on the molecular weight; for example, DEAE-dextran (molecular weight 2×10^6) was most effective at 0.3 $\mu\text{g}/\text{ml}$, whereas spermine tetrahydrochloride (mol wt 348) was best at 400 $\mu\text{g}/\text{ml}$. The concentration optima observed in all experiments were relatively sharp (data not shown), as has also been demonstrated by Melechen, Hudnik-Plevnik, and Pfeifer (19) for protamine sulfate.

Since some variation in the facilitation of transfection, depending on the age of the spheroplasts, was observed (Table 1, column 4), transfection assays were performed in the presence of basic polymers at various times (Table 2). Surprisingly, spermine (which had little or no effect of transfection with 3-h-old spheroplasts [see Table 1]) dramatically increased the efficiency of transfection by T7 DNA with 48- and 72-h-old spheroplasts when protamine sulfate had already lost effect. Also, the effect of spermine on ϕX174 replicative form transfection was minimal, showing that facilitation of transfection by spermine depends on the DNA employed (Table 2). Most fortuitously, adding both protamine sulfate and spermine resulted in the early facilitation of transfection (which is observed with protamine sulfate alone) as well as the stabilization of the spheroplasts (which is observed with spermine alone). Addition of both protamine sulfate and a polyamine was adopted as the new standard procedure for preparing spheroplasts (see Materials and Methods).

Various substances were now tested in combination with protamine sulfate to see if they

TABLE 1. Facilitation of ϕX174 RF transfection by basic polymers with *E. coli* W3350 spheroplasts 1 to 3 h old

Basic polymer ^a	Manufacturer's mol wt	Average facilitation over control	Range of facilitation	Optimum concn ($\mu\text{g}/\text{ml}$)	No. of expt
Protamine sulfate	Heterogeneous	215×	2-1300×	25	18
Filtered protamine ^b sulfate	10 to 50,000	248×	38-902×	6	5
Poly-L-arginine	10 to 20,000	26×	1-145×	0.5	9
Poly-L-lysine	3 to 5,000	20×	3-73×	3	6
Poly-L-ornithine	70,000	19×	2-52×	2	4
Histones		7×	1-9×	15	6
Poly-L-lysine	16,400	5×	4-7×	0.5	4
Poly-L-lysine	75 to 200,000	4×	2-7×	0.5	5
DEAE-dextran	2,000,000	3×	2-6×	0.3	5
Spermine ^c	348	2×	1-4×	400	5

^a All compounds were added during the preparation of the spheroplasts (see Materials and Methods) since later addition produced much weaker effects.

^b Protamine sulfate (Eli Lilly) was passed successively through Amicon filters with molecular weight cutoffs of 10,000 and 50,000. This procedure removed 75% of the biuret-positive material.

^c The following compounds were ineffective in facilitating transfection: heparin, spermidine trihydrochloride, putrescine dihydrochloride, cadaverine hydrochloride; 3,3'-diaminopropylamine; N,N' -diethyl-1,4-pentanediamine; 4-dodecyl-diethylene triamine; tetraethylene pentamine; N,N' -bis (2-aminoethyl)-1,3, propanediamine; N,N' -bis (3-aminopropyl 1,3-propanediamine; 1,1,4,7,7-pentamethyldiethylene triamine; N,N' -bis[(3-(2-amino ethylamine) propyl) ethylene diamine.

TABLE 2. Dependence of facilitation of transfection on the age of the spheroplasts

Transfecting DNA	Age of spheroplasts when assayed (h)	Infective centers found with spheroplasts containing ^a			
		No addition	Protamine sulfate (25 μ g/ml)	Spermine (HCl) ₄ (400 μ g/ml)	Protamine sulfate (25 μ g/ml) plus spermine (HCl) ₄ (400 μ g/ml)
ϕ X174 RF (5 $\times$ 10 ⁴ molecules)	3	56	1,046	50	1035
ϕ X174 RF (5 $\times$ 10 ⁴ molecules)	24	2	18	2	82
ϕ X174 RF (5 $\times$ 10 ⁴ molecules)	48	0	7	2	7
T7 DNA (2.6 $\times$ 10 ⁹ molecules)	3	0	65	119	82
T7 DNA (2.6 $\times$ 10 ⁹ molecules)	24	0	14	231	285
T7 DNA (2.6 $\times$ 10 ⁹ molecules)	48	1	5	434	1,165
T7 DNA (2.6 $\times$ 10 ⁹ molecules)	72	0	0	85	
Lambda DNA (8 $\times$ 10 ⁶ molecules)	3	1	2,260	225	2,500
Lambda DNA (8 $\times$ 10 ⁶ molecules)	24	0	1,403	862	3,500
Lambda DNA (8 $\times$ 10 ⁶ molecules)	48	5	7,000	4,300	10,000

^a All compounds were added during the preparation of the spheroplasts since later addition produced much weaker effects.

could exert a stabilizing effect on the spheroplasts. Table 3 shows that a number of polyamines other than spermine could stabilize spheroplasts for transfection. The following conclusions are drawn from this table and similar results from other experiments. (i) Protamine sulfate gave optimal facilitative effects during the first 10 h, as previously noted (5) and older spheroplasts rapidly lost competence. (ii) For lambda and ϕ X174 DNA transfection, spermine as well as all of the other polyamines listed in the table strongly stabilized spheroplasts for 96 h and longer in the presence of protamine sulfate. (iii) For T5 and T7 DNA transfection, only spermine, *N,N'*-bis (3 aminopropyl)- 1,3-propanediamine, and perhaps spermidine stabilized spheroplasts. Thus the stabilization of spheroplasts varies considerably depending on the DNA used in the transfection assay.

Table 4 presents a summary of recent work on the transfection of *E. coli* spheroplasts. Four important criteria for efficient and reproducible transfection assays are listed: maximum efficiency of transfection, the fraction of spheroplasts yielding maximum efficiencies, the variation in the competence of the spheroplasts, and the stability of spheroplasts for transfection assays.

DISCUSSION

The facilitation of transfection by protamine sulfate shows a remarkably high specificity; only spermine fully substituted for protamine sulfate with some DNAs while 11 other poly-

amines, DEAE-dextran, histones, and polymers of basic amino acids had little or no effect (Table 1). Poly-L-arginine, a close chemical relative of protamine sulfate, was much less effective than protamine sulfate. In other transfection systems, high specificity has been observed: Koch, Quintrell, and Bishop (12) found DEAE-dextran and poly-L-ornithine most effective in stimulating transfection by poliovirus RNA, whereas Aoki and Takebe (1) found poly-L-ornithine effective in facilitating transfection of tobacco spheroplasts by tobacco mosaic virus RNA; Kohn and Green (13) and Green (8) showed that spermine enhances transfection of *Bacillus subtilis* by SP82 phage DNA.

Each basic polymer had a sharp concentration optimum for the facilitation of transfection. Higher-molecular-weight compounds were effective at lower concentrations (Table 1). Furthermore, facilitation of transfection depended on the phage DNA used in the assay (Table 2). Hopefully, these observations will aid efforts to facilitate transfection in other assay systems.

The widespread facilitation of transfection by basic polymers observed in systems as diverse as *E. coli*, mammalian, and tobacco cells raises the possibility that protamines or polyamines are components of natural competence factors. Indeed, Leonard and Cole (16) have isolated a natural competence factor with polyamine- or protamine-like characteristics from *Streptococcus chollis*. Experiments were performed to measure the facilitating effect of protamine

TABLE 3. Stabilization of *E. coli* W3350 spheroplasts by polyamines in the presence of protamine sulfate

Additions to spheroplasts ^a	Infective centers found on two plates using																	
	φX174 RF (5 × 10 ⁴ molecules) with spheroplasts aged for:						Lambda DNA (1 × 10 ⁷ molecules) with spheroplasts aged for:						T5 DNA (2.4 × 10 ⁸ molecules) with spheroplasts aged for:					
	2 h	24 h	48 h	72 h	96 h		3 h	24 h	48 h	72 h	96 h		2 h	24 h	48 h	72 h	96 h	
None	38	11	51	50	55		0	0	0	0	0		0	0	0	0	0	
25 μg of protamine sulfate per ml	413	92	98	85	72		2,210	847	268	16	7		45	3	0	0	00	0
Protamine sulfate plus 400 μg of spermine (HCl), per ml	110	712	681	684	386		1,736	3,374	3,092	3,882	4,204		170	96	97	135	234	26
Protamine sulfate plus 180 μg of N,N'-bis (3 amino-propyl)-1,3 propanediamine per ml	310	329	276	281	263		1,936	4,056	3,788	3,980	3,653		914	462	433	530	485	23
Protamine sulfate plus 250 μg of spermidine (HCl), per ml	654	509	591	164	88		1,960	2,605	1,953	846	2,954		1,824	385	159	50	50	9

^aThe following compounds were nearly as effective as those listed: 260 μg of N,N'-bis[3-(2-aminoethylamine) propyl]ethylene diamine per ml; 190 μg of tetraethylene-pentamine per ml; and 160 μg of N,N'-bis (2-aminoethyl)-1,3-propane-diamine per ml. The following compounds were much less effective than those listed: 3,3' diaminodipropylamine, heparin, N¹,N¹-diethyl-1,4-pentanediamine, 4-dodecylidethylene triamine, putrescine dihydrochloride, cadaverine dihydrochloride, and 1,1,4,7,7 pentamethyl-1-diethylenetriamine. All of the compounds had to be added during spheroplast preparation to be effective.

TABLE 4. *Efficiency of transfection, variations in competence, and stability of competent cells used for E. coli transfection assays*

DNAs used for transfection	Type of competent cell	Maximum efficiency of transfection	Spheroplasts giving maximum efficiency of transfection (%)	Variation in efficiency of transfection	Stability of spheroplasts (h)	References
T1	Lysozyme-EDTA spheroplasts plus protamine sulfate	5×10^{-7}				10
Lambda	Helper-infected cells plus protamine sulfate	10^{-3}		10^{-3} to 10^{-5}		17
Lambda ϕ X174 replicative form	Calcium-shocked cells Lysozyme - EDTA spheroplasts plus protamine sulfate	2×10^{-4} 10^{-1}	10	2×10^{-4} to 2×10^{-5} 10^{-1} to 10^{-3}	10	18 4
Lambda	Lysozyme - EDTA spheroplasts plus protamine sulfate	10^{-3}	10	10^{-3} to 10^{-5}	10	4
fd replicative form	Lysozyme - EDTA spheroplasts plus protamine sulfate	10^{-6}	10	10^{-6} to 10^{-8}	10	4
T7	Lysozyme - EDTA spheroplasts plus protamine sulfate	3×10^{-7}	10	3×10^{-7} to 3×10^{-9}	10	4
T4	Lysozyme - EDTA spheroplasts plus protamine sulfate	10^{-5}	10	10^{-5} to 10^{-7}	10	4
T5	Lysozyme - EDTA spheroplasts plus protamine sulfate	3×10^{-6}	10	3×10^{-8} to 3×10^{-8}	10	4
P22	Lysozyme - EDTA spheroplasts plus protamine sulfate	3×10^{-9}	10	3×10^{-9} to 3×10^{-11}	10	4
T2, T4	Freeze-shocked cells	10^{-8}				11
T4	Penicillin spheroplasts	2×10^{-9}				2
Lambda	Lysozyme-EDTA spheroplasts plus protamine sulfate	1×10^{-5}	20	10^{-5} to 6×10^{-5}	48	19
Lambda	Lysozyme-EDTA spheroplasts plus protamine sulfate	4×10^{-4}			1	24
T4	Lysozyme-EDTA spheroplasts plus protamine sulfate	10^{-6}			1	24
Lambda	Lysozyme-EDTA spheroplasts plus protamine sulfate and polyamine	10^{-3}	40	10^{-3} to 1×10^{-4}	48-120	This paper
T7	Lysozyme-EDTA spheroplasts plus protamine sulfate and polyamine	3×10^{-7}	20	10^{-6} to 3×10^{-8}	24-48	This paper
T5	Lysozyme-EDTA spheroplasts plus protamine sulfate and polyamine	3×10^{-6}	20	3×10^{-6} to 10^{-7}	24-48	This paper

sulfate in the streptococcal transformation system and of the streptococcal competence factor in the *E. coli* transfection system. No facilitation was observed in either case (D. G. Leonard, personal communication; R. Benzinger, unpublished observations). The failure of these experiments might be attributed to the high specificity of transfection factors observed above and the high specificity of streptococcal competence factors (20); they might also be caused by a fundamental difference between the transfection and transformation processes (25). Further studies of the basic proteins in microorganisms should yield interesting results; Kuo and August (14) have already shown that basic proteins are a requirement for phage RNA replicase and that mammalian histones and protamine sulfate will substitute in the reaction.

Previously, our spheroplasts were stable for at most 10 h for transfection assays with large phage DNAs, and only 1 out of 10 preparations gave high competence levels (5). The combination of spermine and protamine sulfate addition to spheroplasts yields stable and highly competent spheroplasts (Table 3). This discovery makes it possible to routinely use the assay in large-scale experiments and to check the reproducibility of results with the same spheroplast preparation.

Spermine may also facilitate transfection as well as stabilize *B. subtilis* competent cells (8, 13). This function is in agreement with the data of Tabor (22), who found that the addition of spermine to spheroplasts greatly stabilized them to osmotic shock. On the other hand, Razin and Rozansky (21) noted that spermine also exerts a toxic effect on *E. coli*. This toxicity is considerably reduced for nonmetabolizing cells, and it is surely no coincidence that our spheroplasts are most stable in minimal medium, a revival of earlier procedures (3). A factor which had not been assessed in this study is the sensitivity of polyamines to oxidation by air or intracellular enzymes (23); the oxidized products may be toxic to spheroplasts. When other polyamines were tested for their stabilizing effect on spheroplasts, several besides spermine (see Table 3) were found to be active; for transfection by lambda and ϕ X174 DNAs as many as six different relatives of spermine were active; however, for T5 and T7 DNA transfection, mainly *N,N'*-bis (3 aminepropyl)-1,3-propanediamine and spermidine but not the other polyamines were effective (Table 3). These results are also in marked contrast to those obtained in the facilitation experiments (Table 1) where only spermine could be substituted for protamine sulfate. Thus, facilitation of

transfection and stabilization of spheroplasts may be entirely different processes, especially since some compounds can only perform one of these two functions. In any case, the beneficial effects of polyamines for transfection of *E. coli* spheroplasts once again point up the importance of polyamines in living systems, as has been extensively documented in a recent book (6) and symposium (9).

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