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Enhancement of Pneumococcal Transfection by Protamine Sulfate

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Protamine sulfate enhanced transfection of *Streptococcus pneumoniae* by DNA of ω3 phage by factors as large as 10^5-fold, provided it was present at the time the cells were added to the DNA. For DNA concentrations well below 1 μg/ml, the optimum amount of protamine sulfate was near 1 μg/ml of cells. Higher DNA concentrations required more protamine for maximum effect, and in all cases transfection fell when protamine was in excess. Transformation was not enhanced by low protamine levels and was inhibited by higher levels. A recipient strain with low but finite endonuclease activity and normal transformability showed higher transfection than did the wild type at low DNA concentrations but less than did the wild type at high DNA concentrations. Protamine sulfate enhanced its transfection at low, but not high, DNA concentrations. The behavior of this strain and the enhancement of transfection by protamine sulfate of wild-type cells were each consistent with less cutting of the donor DNA at the cell surface, which is part of the normal entry process in naturally competent gram-positive bacteria. Less cutting would lead to entry of fewer but longer strands that would be more efficient in reconstruction of the 33-megadalton phage replicon. We suggest that in this system protamine enhances transfection by inhibition of the surface nuclease action that is part of the normal entry process.

Transfection by viral nucleic acids is inefficient but can be strongly stimulated in animal cells (reviewed in reference 4) and in *Escherichia coli* spheroplasts or calcium-shocked cells (1, 2, 5, 6) by a variety of basic polymers, of which protamine sulfate (PS) appears to be most effective. The mechanisms of these effects are still obscure (1).

Transfection of pneumococcus (*Streptococcus pneumoniae*) by the 33-megadalton DNA of phage ω3 is also very inefficient (14); one of the reasons is that the normal entry process in naturally competent gram-positive bacteria involves extensive cutting of donor DNA at the cell surface, followed by entry of single-strand fragments of the original donor (8, 12). To regenerate a replicon, two or more of these must associate into a gapped structure that can be completed by repair synthesis. When the cell surface is only partly saturated with donor DNA, the entering strands have a median length near 7 kilobases (11), and reconstruction of the 50-kilobase phage replicon is unlikely. Porter and Guild (14) found that at 1 μg of DNA per ml, where transformation of chromosomal genes was near maximum, phage DNA gave only a few infective centers (IC) per milliliter. However, above this concentration, transfection rose rapidly, about as the cube of the DNA concentration, and was still rising at over 100 μg of DNA per ml, where over 10^6 IC per ml still corresponded to only 10^{-6} per phage DNA molecule in solution. Other data implied that the biologically effective DNA used the normal entry pathway, and Porter and Guild concluded that the response was due to an increase in the number of longer strands entering as the last remaining cutting sites on the cell surface became totally saturated (14).

We report here that transfection of pneumococcus by ω3 DNA is stimulated as much as 10^5-fold by PS, so that transfection is readily detected at a few nanograms of DNA per milliliter. Transfection falls again above an optimal level of PS that increases with DNA concentrations above 1 μg/ml. We also describe some results with nuclease-deficient recipients and the interaction of these mutations with the PS effect. Transformation was not enhanced by PS, implying that its effect was not to facilitate entry of DNA. Instead, the results suggest that much of the effect in this system may be due to inhibition of the cutting at the cell surface. (A preliminary account of this work was presented at the 82nd

Annual Meeting of the American Society for Microbiology, 7–12 March 1982, Atlanta, Ga. [L. P. Goscin and W. R. Guild, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, H106, p. 130].)

MATERIALS AND METHODS

Phage, bacteria, and DNA. Pneumophage ω3 and recipient strains Rx1, DP1775, and DP1776 have been described (13, 14). Strain DP1700 is described below. Media, buffers, and growth of cells to competence were as described previously (13, 14). Growth and assay of phage stocks and preparation of phage DNA from CsCl-purified phage were as described previously (13, 14), except that the final dialysis of the DNA was against SSC/5 (SSC is, 0.15 M NaCl plus 0.015 M sodium citrate).

Strain DP1700 was constructed in this laboratory by N. Shoemaker (personal communication) by selection for the Noz phenotype (no zone on a methyl green-DNA agar plate [7]) after transformation of Rx1 by DNA from an R6 *end-1 exo-3* strain received from S. Lacks; DP1700 gives no zone of hydrolysis of DNA around its colonies, even after many days of incubation. By this criterion, it appears to lack both the major endonuclease and the exonuclease described by Lacks (7) and is presumably *end-1 exo-3*. In vitro assays by A. Rosenthal and S. Lacks (personal communication) showed levels of endonuclease typical of *end-1* strains, that is, 2 to 10% of wild type (10). DP1700 transforms as well as do wild-type strains, as do other *end-1* strains (10).

Chemicals. PS from salmon sperm (Nutritional Biochemicals Corp., Cleveland, Ohio) was suspended in sterile deionized water and stored at 4°C. Pancreatic DNase I and trypsin were from Worthington Diagnostics, Freehold, N.J. The trypsin was put into solution immediately before use.

Preparation of labeled calf DNA. Ten units of DNA polymerase alpha from rabbit bone marrow (3; L. P. Goscin and J. J. Byrnes, manuscript in preparation) were incubated with 0.4 mg of calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) in 1.0 ml (final volume) of 40 mM morpholinepropanesulfonic acid (pH 6.75), 10 mg of bovine albumin per ml, 0.4 mM $MnCl_2$, 19% (vol/vol) glycerol, 100 mM KCl, 0.8 mM GMP, 10 μM each of dGTP, dATP, and dCTP (P-L Biochemicals), and 1.1 μM [^{3}H]dTTP (78 Ci/mmol; New England Nuclear Corp., Boston, Mass.) at 37°C for 1 h. EDTA was then added to 20 mM, and the reaction mix was heated for 5 min at 80°C before being stored frozen.

Reactions with PS: transfection and transformation. Except where noted, incubations of donor DNAs with PS and other reagents were done in total volumes of 100 to 130 μl of SSC/5, to which 0.9 ml of competent cells was added. After 30 min at 37°C, exposure to DNA was terminated by the addition of DNase I; the cultures were then diluted and plated either for transformants or for IC (before lysis) as in a standard plaque assay (13, 14). A maximum of 0.3 ml of undiluted culture could be plated to give a good lawn with visible plaques. For low levels of transfection, multiple platings were made. For high levels, where the culture had been diluted, 0.1 ml of uninfected culture was added to give a lawn.

The concentrations of DNA and PS given here are those in the mixture after the addition of the cells. It may be important that the concentrations of each were about 10-fold higher during the preincubation period. Except where noted otherwise, the time of preincubation was 30 min.

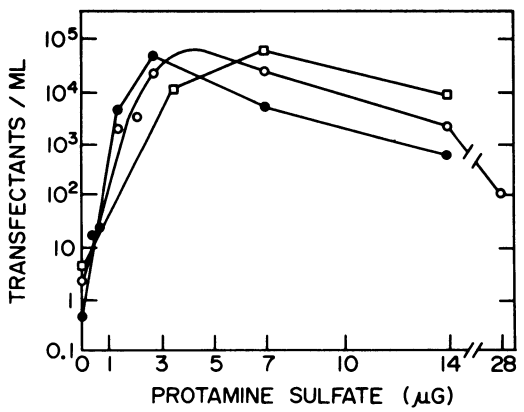

FIG. 1. Effect of PS on transfection of Rx1 cells by three concentrations of phage DNA. The abscissa corresponds to PS per milliliter of cells after addition of the cells to the PS-DNA mixture. Preincubation was for 30 min at 37°C. DNA per milliliter of cells was 0.3 μg (●), 1.2 μg (○), or 4.8 μg (□).

RESULTS

Concentration effects. When PS was added to donor DNA before exposure to competent cells, transfection rose rapidly as the amount of PS increased to an optimum level, beyond which transfection fell again (Fig. 1). For 0.3 μg of DNA per ml of cells, the addition of 2.8 μg of PS led to 4.5×10^4 IC per ml, whereas in its absence one plaque was seen in 2.1 ml of cells plated. The factor of enhancement was near 10^5. For more DNA, more PS was needed to reach maximum, and results at even higher DNA levels showed optimal weight-to-weight ratios a little over 1, perhaps reflecting the expected charge equivalence binding of protamine to DNA (see reference 18). However, although this probably is one component of the reaction, closer examination showed that the optimum ratio increased as DNA fell, as for 0.3 and 1.2 μg of DNA in Fig. 1.

A plot of optimum PS against DNA (Fig. 2) extrapolated to an intercept between 1 and 2 μg of PS, suggesting that the optimum would be in this range even for much lower DNA. This was confirmed by experiments of the kind shown in Fig. 3 for 0.05 μg of DNA. In the absence of PS, no plaques were expected, and none were seen in 0.9 ml of cells plated. The addition of PS led to 10^4 IC per ml at 0.7 to 1.1 μg of PS per ml, followed by a slow fall as PS increased further. Here also the factor of enhancement was near

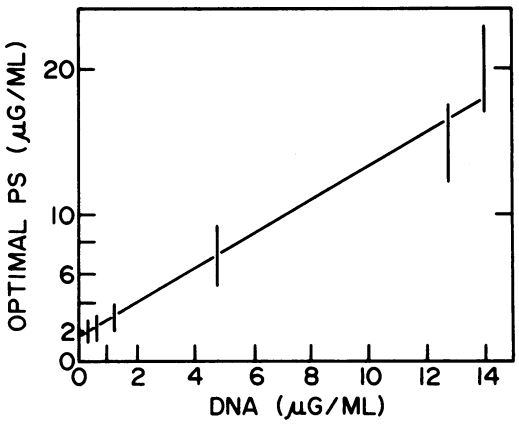

FIG. 2. Optimal PS as a function of DNA concentration. The line as drawn has a slope near 1.2 and an intercept near 1.5.

10^5 as observed and may have been higher compared with the expected value.

Time of action of PS. It was possible that PS altered the processing of the DNA by acting on the cells after entry. However, the addition of PS after the cells had been added led to little or no enhancement of transfection (Fig. 4), implying that the protamine had to be present during or before entry. The small enhancement seen at 5 and 10 min was consistent with that expected if some of the DNA initiated a reaction with the cells after this time. When PS was added very near the time of addition of the cells, the results were erratic (not shown), probably, but not certainly, due to mixing or kinetic problems. Although 60 min of preincubation was too long, results were fairly reproducible with preincubation times between 45 and 10 min, and 30 min was chosen for most experiments. A variety of kinetic effects could be important for a more detailed analysis of the reactions occurring.

Reversal by trypsin. Digestion of PS-DNA mixtures by trypsin before addition of cells gave over 99% reversal of the enhancement (not shown). Therefore, the effect required the PS to be present at the time of uptake and was not due to either a trace enzyme acting on the DNA or to a nonpolypeptide factor in the preparation.

Effect of PS on transformation. Optimal or suboptimal levels of PS for transfection had no effect on transformation of a chromosomal gene (Fig. 5A), and higher levels of PS inhibited it (Fig. 5B and C). Because the numbers of total colony-forming units were unaffected (not shown), the inhibition was not due to cell killing. These results suggested that the enhancement of transfection was not due to a quantitative increase in the weight of entering DNA but rather to a qualitative change in the DNA.

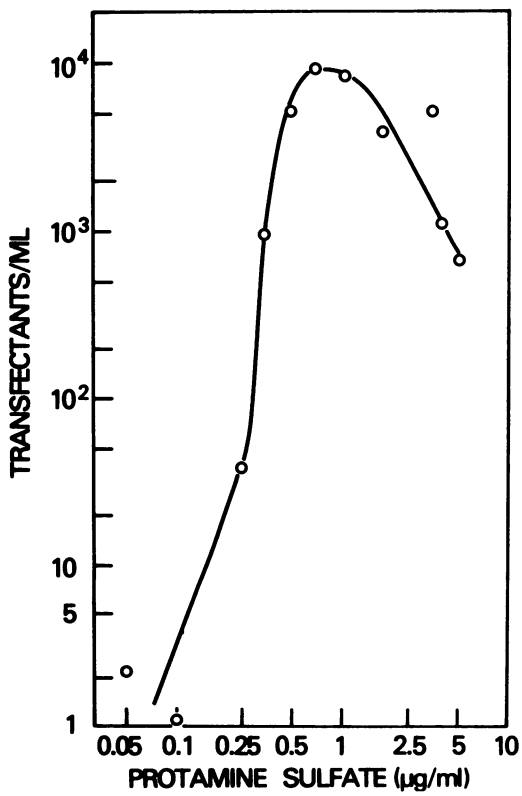

FIG. 3. Transfection of Rx1 by 0.05 μg of DNA in the presence of PS. For 0, 0.05, and 0.1 μg of PS, 0, 2, and 1 plaque was seen in 0.9 ml of cells plated.

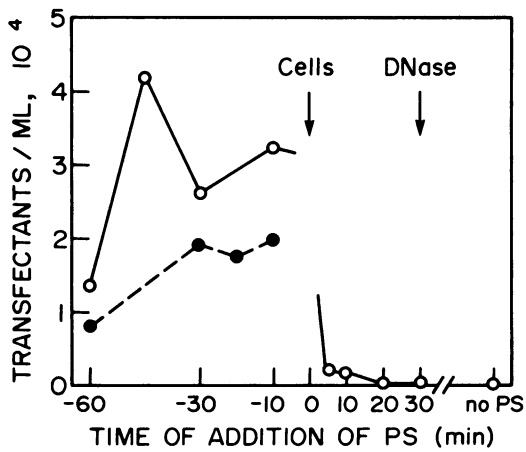

FIG. 4. Dependence on time of addition of PS. At the times indicated, relative to the addition of 0.9 ml of cells, 3.5 μg of PS was added to 1.0 μg of DNA, followed by gentle mixing (○). The points for addition at 30 min and for no PS represent 30 and 19 plaques per 0.9 ml of cells, respectively. The filled symbols represent another experiment with fewer time points.

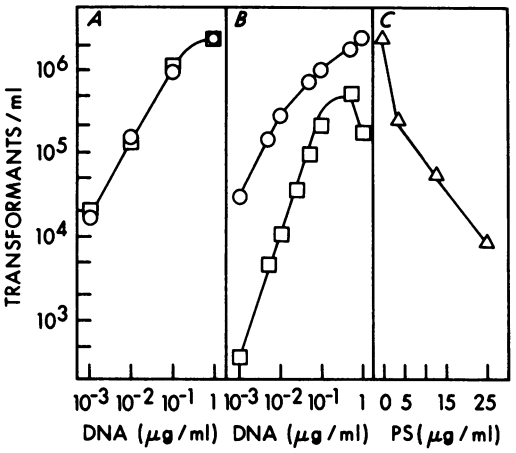

FIG. 5. Effect of PS on transformation of a chromosomal gene. (A) Donor DNA was preincubated with 1.7 times its weight (□) or with no (○) PS. (B) 3.5 μg (□) or no (○) PS was added to each DNA concentration. (C) 1.0 μg of DNA was incubated with increasing amounts of PS. The donor DNA was from a streptomycin-resistant derivative of Rx1.

Dose-response with optimum PS. Given the above results, we examined the dose-response curve over a range of low concentrations of DNA using both 1.1 and 3.5 μg of PS per ml, at or just above the optimum. Figure 6 shows these results along with those for several higher DNA concentrations, for which more PS was added as indicated. For comparison, some typical data for transfection in the absence of PS are included.

In the low DNA range, 1.1 and 3.5 μg of PS gave curves of similar shape that were consistent with Fig. 3 in showing that 3.5 μg of PS was a little beyond optimum up to 1.0 μg of DNA, where 3.5 μg of PS gave higher transfection than did 1.1 μg. At higher DNA concentrations, the curve continued upward, provided more PS was added. The shape in this region should not be considered reliable because it was difficult to be certain that the PS was truly optimal. However, as the DNA concentration increased, the factor of enhancement decreased.

For low DNA concentrations, transfection was detectable at 2.5 ng of DNA per ml, and from this value up to about 100 ng/ml the slope of the curve was near 2 (Fig. 6). In another experiment, 1.2 μg of DNA was incubated with 3.5 μg of PS before dilution in two-fold steps from 2- to 16-fold and exposure to competent cells; the transfectants fell on a curve with a slope of 1.9 (not shown). In a third experiment at 1.5 μg of PS per ml, the slope was near 2 over the range from 5 to 200 ng of DNA per ml. Thus, at optimal PS levels, collision of two donor particles with the cell was needed to give an IC.

Aggregation by PS. The addition of one- to twofold excess PS to a few micrograms of DNA often led to immediate formation of visible stringy aggregates, and it was under these conditions that transfection or transformation was reduced. To examine this further, increasing PS was added to labeled calf DNA, the mixtures were centrifuged, and the supernatants were assayed for the presence of DNA. The results in Table 1 correspond to the use of 3.7 μg of DNA per ml of cells in the transfection assay and confirm that aggregation occurred at PS/DNA

TABLE 1. Aggregation of DNA by PS[a]

PS (μg)	PS/DNA (μg/μg)	DNA in supernatant	
		cpm	%
0	0	4,347	100
7.5	0.28	3,728	86
17.5	0.47	2,621	60
25	0.68	1,070	25
50	1.35	505	12
100	2.7	404	9

[a] Samples containing 37 μg of ^{3}H-labeled calf DNA and the indicated amounts of PS in 1.0 ml of SSC/5 were incubated for 10 min at 37°C and then centrifuged for 5 min at 7,000 rpm in an SS34 rotor. The supernatants were precipitated with trichloroacetic acid and counted in a liquid scintillation counter.

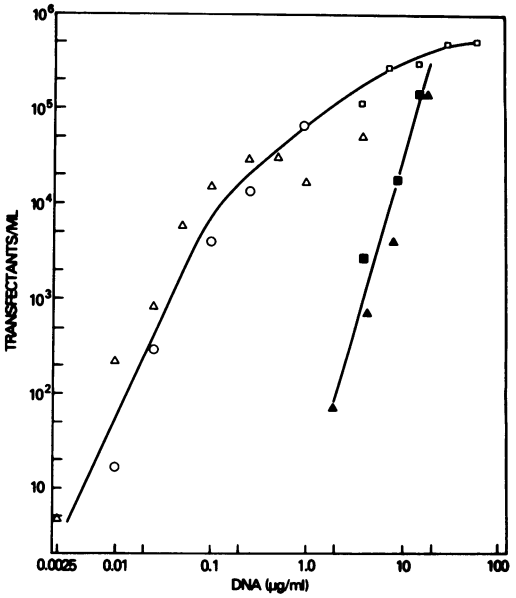

FIG. 6. Dose-response for transfection at or near optimal levels of PS. Symbols: △, 1.1 μg of PS; ○, 3.5 μg of PS. For the five highest DNA levels (□), 7.5, 12.5, 12.5, 25, and 25 μg of PS was added, respectively. Filled symbols represent results in the absence of PS.

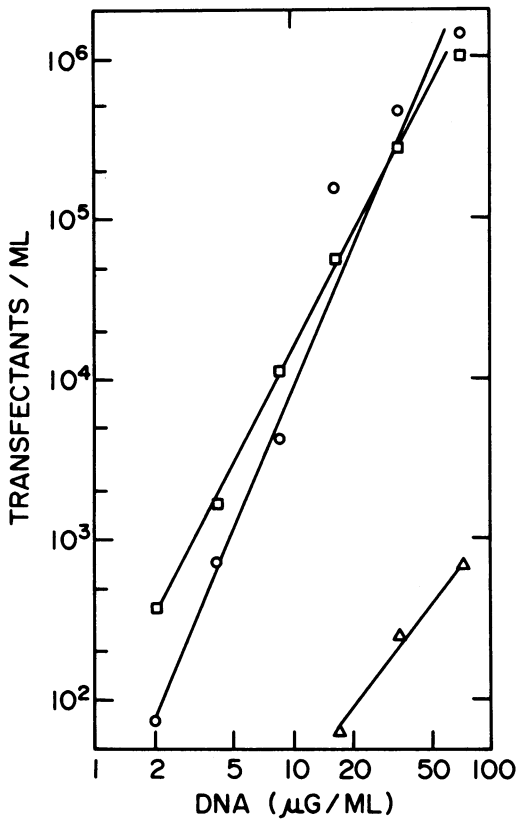

FIG. 7. Dose-response for transfection of Rx1 (○), DP1700 (□), and DP1775 (△) in the absence of PS. In these cultures, 1.0 μg of cell DNA gave 1.9×10^6, 1.9×10^6, and 2.5×10^5 Str^r transformants per ml, respectively.

ratios even below the optimum level for enhancement of transfection. At least part of the fall above the optimum may simply reflect the fact that DNA was less accessible to the cells.

Transfection in endonuclease-deficient strains and their interaction with PS. Strains carrying the *end-1* mutation have a reduced level of the membrane endonuclease associated with the normal entry path for donor DNA, but transform as well as wild-type strains (10). (A report that the endonuclease was not needed at all for transformation [16] has been withdrawn [see Discussion]). In other End$^-$ strains, transformation is reduced and transfection is reduced much more strongly, by about the cube of the effect on transformation; these results plus a general requirement for competence led Porter and Guild to conclude that transfection required the entry of an average of three strands by the endonuclease-dependent pathway (14). The report by Ronda et al. (15) that transfection by Dp4 phage DNA was better in a strain deficient in endonuclease seemed to contradict this conclusion, but in fact it did not because their strain carried *end-1* and was normal in transformation. In preliminary work, R. D. Porter (personal communication) had observed that the *end-1 exo-3* strain DP1700 (see above) gave more transfection than did Rx1 at low DNA concentrations. However, because it gave normal levels of transformation, Porter set it aside in favor of developing a set of strains in which transformation was deficient due to endonuclease mutations (14).

We examined DP1700 further, and the results seem relevant to the action of PS. For cultures that were equally competent for transformation, DP1700 consistently gave several-fold more transfectants than did Rx1 at low DNA concentrations but lost this advantage at higher DNA levels. Figure 7 is typical of a number of response curves showing slopes of 2.0 to 2.4 for DP1700 and 3.0 to 3.5 for Rx1. The curves crossed one another when the cells were equal for chromosomal transformation. The response of DP1775 in Fig. 7 was similar to that reported for this and other mutants (14); transformation was 0.13 of that in Rx1, and transfection was reduced about 1,000-fold.

The addition of PS enhanced transfection of DP1700 by low concentrations of DNA but had little effect at higher DNA levels (Fig. 8). At 0.05

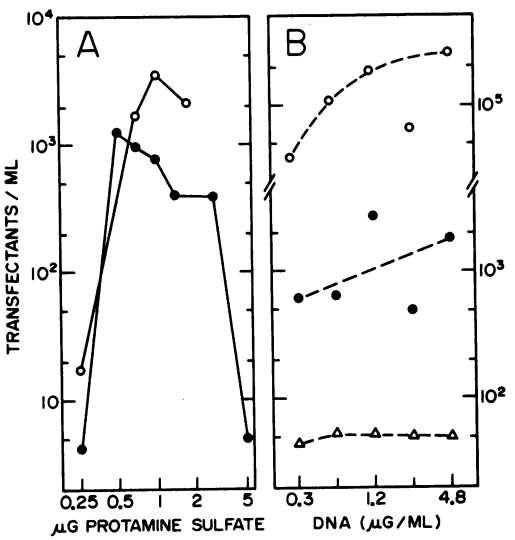

FIG. 8. Response of endonuclease-deficient strains DP1700 and DP1776 to PS. (A) 0.05 μg of phage DNA was incubated with PS and exposed to DP1700 (●) or Rx1 (○) cells. (B) Higher DNA concentrations plus PS (3.5 μg of PS for 0.3 to 2.4 μg of DNA; 7.0 μg of PS for 4.8 μg of DNA) exposed to Rx1 (○), DP1700 (●), and DP1776 (△) cells. Note the break in the ordinate scale. Str^r transformants per milliliter observed in the various cultures were: A, 2.2×10^6 and 2.5×10^6 for Rx1 and DP1700, respectively; B, 2.7×10^6, 1.2×10^6, and 1.6×10^5 for Rx1, DP1700, and DP1776, respectively.

μg of DNA, enhancement was large for DP1700 but less than that seen with Rx1 at the same time; also, the optimum amount of PS seemed to be a little lower for DP1700 (Fig. 8A). With increasing DNA concentrations, Rx1 showed its usual strong enhancement, but for both DP1700 and DP1776 (a strain similar to DP1775), transfection remained roughly constant with DNA and 100- to 1,000-fold lower than in Rx1 (Fig. 8B). In these cultures, DP1700 gave only 0.44 as many transformants as did Rx1 (see the legend to Fig. 8); correcting by the square or cube of this factor still implies 10- to 20-fold lower transfection of DP1700 in the presence of protamine at DNA concentrations at which it gives more transfection than does Rx1 in the absence of protamine (compare Fig. 7). Thus, protamine appeared to enhance transfection of endonuclease-deficient strains at low DNA concentrations but to lose much of this ability as DNA levels increased.

DISCUSSION

Optimal levels of PS present at the time cells were added enhanced transfection of pneumococcus by a 33-megadalton phage DNA by factors as large as 10^5 for low DNA concentrations (Fig. 1 and 3). About 1 μg of PS per ml of cells was required for maximum transfection by DNA concentrations much less than 1 μg/ml, but above 1 μg of DNA per ml, the optimal level of PS increased (Fig. 2).

Possible mechanisms. The fall of transfection above the optimum level of PS was probably due at least in part to aggregation, but a second possibility is discussed below. For the enhancement at lower levels of PS, four mechanisms need consideration: (i) entry of more DNA by the normal pathway, (ii) creation of a new entry pathway, (iii) limited aggregation of the DNA into particles able to donate the multiple strands needed for reconstruction of the phage replicon in a single collision event, and (iv) inhibition of the cutting that normally occurs on the cell surface.

The fact that transformation of chromosomal genes was not enhanced by low to optimal levels of PS and was only inhibited at higher levels (Fig. 5) implies that entry by the normal pathway was not facilitated. PS might create a new pathway, for example, if PS-DNA complexes could penetrate the cell membrane with the DNA in double-stranded form. However, the requirement for the endonuclease for transfection even in the presence of PS (Fig. 8B) makes it unlikely that a new pathway is involved. Thus, both (i) and (ii) seem excluded as major contributors to enhancement by PS.

At low levels of PS, limited aggregation could give particles able to donate two or more strand segments in a single collision event, as does replicating phage DNA extracted from infected cells (14). However, response curves at optimal PS levels rose with the square of the DNA concentration when the protamine was added either before or after the dilution of the DNA (Fig. 6 and discussion above). A requirement for two particles to collide with the cell would suggest that aggregation is unlikely to be the major cause of the enhancement by protamine, although it may contribute some of the effect.

In this system, transfection by a large DNA should be enhanced by any reaction leading to fewer cuts being inserted into the donor DNA on the cell surface before entry. During or shortly after binding, the DNA receives single-strand nicks from an otherwise uncharacterized activity (8, 9). In *end*$^+$ cells, double-strand cuts appear rapidly, apparently by the endonuclease cutting the other strand near the first nick, followed by degradation of one strand and entry of the other (8, 11, 12). If PS were to inhibit either or both of these cutting activities, longer strands should enter and be much more likely to regenerate a large replicon, quite possibly by the two-hit process implied by the slope of the response curve. As long as entry was not totally blocked, the total weight of DNA entering could be the same as that in the absence of PS, in the form of fewer but longer strands. On this model, part of the inhibition at high PS levels could be due to direct blocking of entry.

Thus, by exclusion of alternate hypotheses and on its own merits, inhibition of nuclease action seems likely to be the major cause of enhancement of transfection by PS in the pneumococcus system. This is one of the hypotheses suggested for its action in *E. coli*, but data in that system do not seem to support it (1). Other systems could, of course, differ from the pneumococcus system.

Endonuclease-deficient recipients. The transfection response of DP1700 and the different reaction of this strain to PS were of interest. Although it is difficult to exclude effects of further unknown mutations in DP1700, the observation of Ronda et al. that transfection by another phage DNA was better in an *end-1* recipient than in the wild type (15) suggests that this mutation may account for much of the behavior of DP1700. Strains carrying *end-1* give normal transformation, even though extracts show at most 10% of wild-type nuclease activity. Seto et al. (16) reported that some isolates from a heavily mutagenized *end-1* culture had little or no endonuclease and unusual transformation properties, in that they failed to become competent spontaneously at the same time that the parent did, but were fully transformable when

activated by competence factor. These workers suggested that the endonuclease was unrelated to transformation. However, on testing two of the strains of Seto et al., Lacks found typical *end-1* levels of the nuclease (9). Seto et al. then found that Lacks's extreme End$^-$ mutants, *end-14* and *noz-19*, could not be activated to competence, and they now agree that the defect in their strains is not due to the lack of the endonuclease (17). The difference in the spontaneous development of competence could be a matter of timing, inasmuch as they did not transfer the defect from the mutagenized culture and tested for competence at only one time point. We have seen strains that were "nontransformable" ($<10^{-5}$) at the usual time in the competence cycle but became fully competent 45 min later (N. B. Shoemaker and W. R. Guild, unpublished data).

A hypothesis like that for the action of PS on wild-type cells can account for the behavior of DP1700. If it has fewer active nuclease molecules than Rx1, but enough to allow for entry of two or more DNA strands per cell, then DP1700 should take up fewer but longer strands. When the total weight of DNA entering is comparable to that in Rx1, equal numbers of chromosomal transformants are expected. Most important, this process could lead to significantly more strands at least half a phage genome long than occur in Rx1 at low DNA concentrations and could generate response curves with slopes near 2, as seen in Fig. 7. At higher DNA concentrations, the competition for cutting sites is thought to increase the mean length of strand entering, and one expects the fewer entry sites on DP1700 to become limiting.

If enhancement by PS and the behavior of DP1700 each reflect less cutting of the DNA, less effect of PS on DP1700 is expected. The results showed that enhancement still occurred at low DNA concentrations, although less than in Rx1 and perhaps at lower PS levels, but that this effect was lost as DNA increased (Fig. 8). The fact that DP1700 gave many fewer transfectants than did Rx1 at higher DNA levels in the presence of PS, whereas it gave more in its absence (Fig. 7), suggests that the protamine may have totally blocked some entry sites. Such an effect could also produce the lower enhancement and the shift toward lower optimal PS level seen at 0.05 μg of DNA in Fig 8A. Thus, enhancement appears to reflect a balance between increasing length and decreasing number of entering strands.

Implicit in the above hypothesis is the prediction that these effects should be less for smaller replicons. C. W. Saunders has examined the effect of PS on plasmid transformation and has found enhancement for the 20-megadalton pIP501 but not for the 3.5-megadalton pMV158 (Saunders and Guild, in preparation).

Where does the PS act? If inhibition of nuclease action is the major reason for enhancement of transfection in this system, is this because the PS coats the DNA, or because it acts directly on the surface nucleases? The most relevant observation is that at low DNA levels about 1 μg of PS per ml of cells is needed, far greater than the amount needed for stoichiometric binding of protamine to DNA. At higher levels of DNA, the optimum PS rises about as expected if the DNA bound 1 to 1.4 times its weight of PS and left a smaller amount free in solution (Fig. 2). In other work (to be published), we have found that pancreatic RNase A can enhance transfection about 20-fold when conditions are such that the effect can be correlated with its binding to DNA. We have also observed 2- to 40-fold enhancement by several polyamines, but have not studied them in detail. It may be that coating the DNA with a basic material gives modest enhancement and that for PS a second action on the cell surface gives further enhancement.

ACKNOWLEDGMENTS

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