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Protoplast Formation and Leakage of Intramembrane Cell Components: Induction by the Competence Activator Substance of Pneumococci

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Treatment of pneumococci with activator (a protein that induces bacterial "competence" to absorb deoxyribonucleic acid molecules and undergo genetic transformation) can cause either protoplast formation or leakage of intracellular components to the medium depending on postincubation conditions. The leaked intracellular components include nucleoside phosphates, β-galactosidase, deoxyribonuclease, autolysin, and hemolysin. Leakage and protoplast formation are induced by the electrophoretically pure activator, and these phenomena require the same conditions as induction of competence for genetic transformation, namely, genetic capacity for competence, protein synthesis, incorporation of choline, and the optimal pH for activation. It is suggested that the activator protein accelerates a normal process of transport (leakage) of autolysin molecules into the periplasmic space. The activity of these autolysin molecules from within would then unmask deoxyribonucleic acid binding sites located on the plasma membrane.

In the competent state, bacteria can absorb deoxyribonucleic acid (DNA) molecules from their environment and undergo genetic transformation. In pneumococci, this physiological condition is induced by a specific macromolecular agent, the activator protein. Upon collision with the cells, the activator seems to attach to receptors located on the plasma membrane. Subsequent to this attachment, the cells develop the capacity to bind DNA molecules provided that some additional requirements are met, namely, synthesis of a new protein (or class of proteins) and incorporation of choline into the cell wall growth zone (31). In the search for the biochemical mechanism of action of the activator, we observed two novel phenomena: induction of protoplast formation, under appropriate conditions, by activator treatment of pneumococci, and leakage of intracellular components to the medium.

MATERIALS AND METHODS

Diplococcus pneumoniae R36A and its derivatives were used in all experiments. Strain RA7 is a clonal isolate defective in some step of DNA binding (32). Strain *cwl* is an autolysin-defective transformant that is not lysed by detergents (10); it was obtained by S. Lacks by transforming competent cells of the *hex* strain with DNA isolated from an autolysin-defective mutant. Stock cultures of both the *hex* and the *cwl* strain were kindly provided by S. Lacks, Brookhaven National Laboratories. The composition of growth media (C and Cd_{en}), the methods used to determine bacterial viability and transformation, and the procedures for preparing activator and transforming DNA are as published previously (7, 34, 36). Electrophoretically homogeneous activator was provided by S. Plotch of our laboratory. For radioactive labeling, bacterial cultures were grown in Cd_{en} medium supplemented either with [6-^{3}H]uridine (50 μCi/ml, 5 μg/ml) or [U-^{3}H]phenylalanine (25 μCi/ml, 5 μg/ml). Growth was measured with a Coleman nephelometer. The composition of SPA solution is as follows: 0.15 M NaCl; 0.05 M potassium phosphate buffer (pH 8); 0.3 mM $CaCl_2$; 10 mM $MgCl_2$; 0.4 mg of albumin per ml; and 2 mg of glucose or sucrose per ml.

Enzyme assays. All enzymes were assayed at 30 C for 16 h. β-Galactosidase was determined with *O*-nitrophenyl-β-D-galactoside at pH 8.0. To 0.5 ml of the supernatant fluid from a bacterial suspension were added 0.8 ml of SPA solution and 0.2 ml of 14 mM *O*-nitrophenyl-β-D-galactoside. Enzyme activities are expressed in arbitrary units of absorbance at 410 nm. For the assay of hemolysin, 0.5 ml (packed volume) of fresh sheep blood cells was suspended in 10 ml of 0.15 M NaCl; 0.5-ml portions of this suspension were incubated with the bacterial extracts (0.5 ml), and the released hemoglobin was determined (optical density at 541 nm) in the supernatant fluids after centrifugation. For assay of deoxyribonuclease, 0.3 ml of DNA labeled biosynthetically with [^{3}H]thymidine (6×10^6 dpm per μg of DNA) was incubated with the bacterial extracts (0.3 ml), and the increase of soluble radioactivity was determined after precipitation with cold 10% trichloroacetic acid and added carrier al-

bumin. Autolysin was assayed with pneumococcal cell walls labeled with [^{3}H]choline as substrate, and the release of non-sedimentable radioactivity was measured after the cell wall suspensions were centrifuged at 10,000 × *g* for 5 min (14).

The total cellular content of these enzymes was determined by using crude, sonically disrupted extracts from cell suspensions of known cell concentration. Enzyme activities leaked into the medium and total enzyme contents were compared on the basis of cell concentrations. In estimating the enzyme activities leaked into the medium, it was necessary to sterilize, by membrane filtration (Millipore Corp.; HA, 0.45 μm), the culture supernatant fluids or to centrifuge them a second time to complete the removal of bacteria. Analysis of the supernatant fluids containing radioactive uracil-labeled compounds was carried out as follows. After precipitation with the cold 10% trichloroacetic acid (in the presence of carrier albumin), the acid-soluble fraction was extracted three times with equal volumes of ethyl ether; residual ether was removed from the water phase by flushing with nitrogen gas. A portion of the trichloroacetic acid-free water phase was treated with intestinal phosphatase (100 μg/ml; Worthington Biochemicals Corp.) at pH 8 and 37 C for 1 h. Both treated and untreated extracts were applied to thin-layer chromatography plates (silica gel, Eastman Kodak Co.), chromatographed in a solvent system of isopropanol-1% NH_4OH (70:30, vol/vol) (6), and compared to standard nucleoside derivatives. The amounts of radioactive compounds moving with different R_f values were evaluated by cutting the chromatogram into rectangles (0.5 by 1 cm) and determining radioactivity in a scintillation spectrometer (Mark II, Nuclear-Chicago). For phase-contrast microscopy, we used a Zeiss R.A. microscope equipped with a planachromat 100/1.25 oil immersion phase-contrast objective and with 1.25× binocular lenses. Photographs were taken on 35-mm Kodak Panatomic X film. A Hitachi HU 11-C-1 electron microscope was used at 75 keV. Pneumococci were fixed with 2.5% glutaraldehyde at room temperature for 30 min followed by postfixation in osmium tetroxide. Epon was used as embedding material.

RESULTS

Activator-dependent protoplast formation. The design of the experiment used to demonstrate activator-dependent protoplast formation is shown in Fig. 1. The bacteria were grown in the chemically defined medium (Cd_{en}) at 37 C and at low pH (pH 6.6) to prevent expression of competence (35) (phase 1). When the cell concentration reached approximately 2 × 10^7 viable units per ml, the pH of the culture was shifted to 8.0 (either by addition of alkali or by centrifugation and resuspension in fresh Cd_{en} medium at pH 8.0). After treatment with the activator (100 U/ml) at pH 8.0 and 30 C for 20 to 30 min (phase 2), the bacteria were centrifuged and suspended in a casein hydrolysate medium (C medium, pH 8.0) containing 20% sucrose, and incubated at 37 C (phase 3).

The design of the experiment was modified when bacteria with ethanolamine-containing cell walls were used (14). In these experiments, phase 1 bacteria were grown in Cd_{en} medium containing ethanolamine instead of choline at 37 C and pH 8. At the beginning of phase 2, still at 37 C, the culture received choline; 1 to 3 min later the culture was transferred to 30 C and activator was added. The rest of the experimental design was unchanged. Experimental variables (pH, addition of chloramphenicol, etc.) were introduced in phase 2 of the experiments, and the extent of protoplast formation was determined by phase-contrast microscopy after various intervals of incubation (10, 30, and 60 min) in phase 3.

Table 1 summarizes the results of a series of

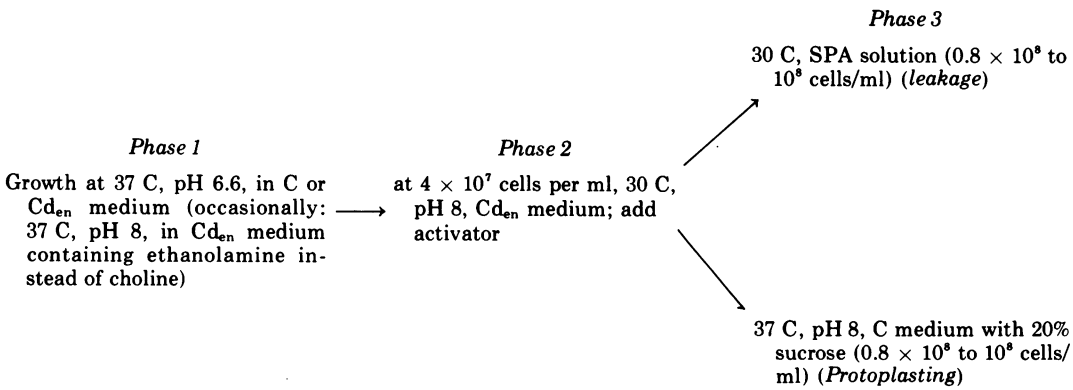

FIG. 1. *Design of experiments that show cellular leakage and protoplast formation. Bacteria were incubated in the appropriate media for various periods. Transfer of cells from one phase of the experiments to the other one was accomplished either by membrane filtration (Millipore Corp.; 0.45 μm) or by centrifugation (5,000 × g, 5 min).*

experiments on protoplast formation. It can be seen that all experimental conditions that typically prevent activation of pneumococci to competence also prevent the appearance of protoplasts in phase 3. These conditions include activator treatment at low pH (35), presence of chloramphenicol (29), ethanolamine-containing cell walls, lack of amino alcohol (28), genetically incompetent bacteria (RA7), and trypsin pretreatment of the activator preparation. Extracts from incompetent cells induced no protoplast formation, whereas the activator purified to electrophoretic homogeneity did.

In most experiments, conversion to protoplasts was complete (100%) within 10 to 40 min of incubation in the phase 3 medium. Protoplast formation was most rapid at 37 C in C medium containing high (20%) sucrose. Lower temperatures retarded protoplast formation, and there were no observable protoplasts in phase 3 at 0 C or if the cells were incubated in 20% sucrose solution from which the growth medium components were omitted.

Figure 2 shows the dramatic difference be-

TABLE 1. *Effect of experimental conditions on the activator-dependent protoplast formation*

Conditions in phase 2		% Cells converted to protoplast after 30 min in phase 3 (%)
Strain, pH	Additions	
Strain R6, at pH 8	None	0
	Activator	100
	Electrophoretically homogeneous activator	100
	Chloramphenicol + activator (100 μg/ml)	1
	No choline + activator	10
	Trypsin-treated activator	0
	Extract from incompetent cells	0
	Activator + DNA	100
	Activator in ethanolamine medium, pH 8	0
	Activator in ethanolamine medium + choline	100
at pH 6.6	Activator	0–10
at pH 8	Activator at 0 C in phase 3	1
at pH 8	Activator; no-growth medium, only 20% sucrose (water) in phase 3	0
Strain RA7, at pH 8	Activator	0

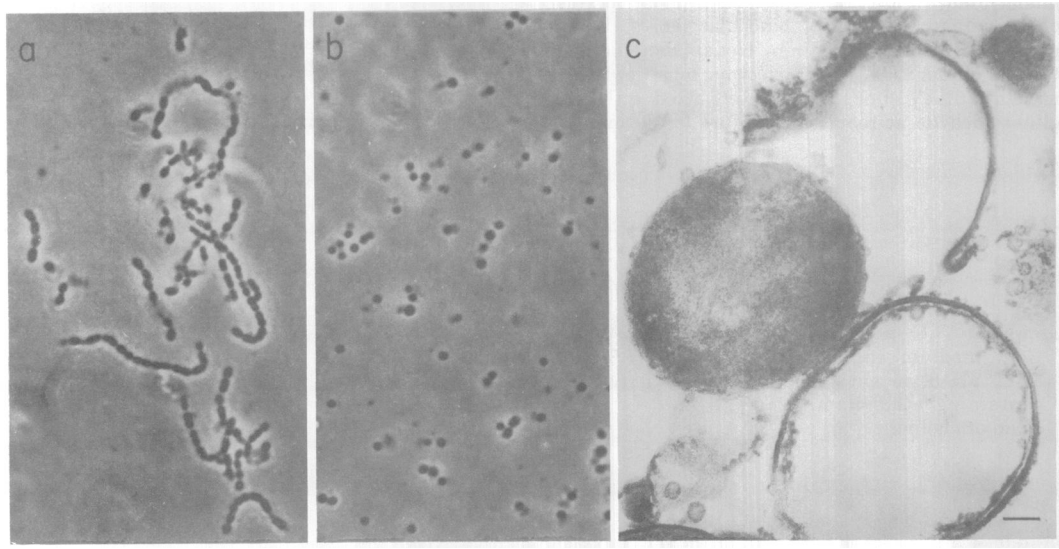

FIG. 2. *Activator-induced protoplast formation. Bacteria were grown in ethanolamine medium (phase 1); they received choline at the beginning of phase 2 of the experiment, with or without (control) the addition of activator. (A) Control and (B) activator-treated bacteria after 30 min of incubation in the phase 3 medium. (C) Electron micrograph of protoplasts from the phase 3 medium. Magnification: ×112,000. Bar indicates 0.1 μm.*

tween competent (activated) and incompetent cells after 30 min of incubation in phase 3 medium. The virtually complete loss of cell wall during protoplast formation of activated cells was confirmed by electron microscopy (Fig. 2C). In this experiment, ethanolamine cells were treated with choline and activator for 20 min. Under these conditions, a maximum of only 15 to 20% of the cell wall is lysis sensitive (i.e., contains choline). In the electron micrographs, one can observe many large, hemispherical wall fragments, obviously representing the "old," lysis-resistant (i.e., ethanolamine-containing) cell walls (13). These observations suggest that the protoplasts emerged after a limited digestion of the equatorial, lysis-sensitive area of the cell walls by autolysin molecules acting from within.

In several experiments we compared the fine structure of competent (activated) and incompetent pneumococci at the end of phase 2 by serial sectioning and electron microscopy. The design of these experiments was similar to that listed on line 2 in Table 1, except that the bacteria were fixed with glutaraldehyde at the end of phase 2. Occasionally, but not always, we noted evidence for limited autolysin action at the cellular equator in competent cells (but never in incompetent bacteria) (Fig. 3).

Activator-dependent leakage. The experimental design for detecting cellular leakage is shown in Fig. 1. After activation, the bacteria were transferred to a buffered salt solution (SPA) and, after various incubation periods, portions of the suspension were centrifuged. The supernatant fluids were used to determine the presence of various enzyme activities or radioactivity. In some experiments, the bacteria were prelabeled by growth in an isotope-containing medium in phase 1. Table 2 summarizes the results of several experiments; it can be seen that activated cells released 3 to 14% of a variety of cellular enzymes into the medium. Genetically incompetent cells (RA7) did not respond this way to the activator treatment, and autolysin-defective cells (*cw1*) seemed to release only radioactive uracil-labeled compounds, but no enzymes, to the medium. Table 3 demonstrates that leakage of β-galactosidase has additional requirements (besides the activator) that coincide with the conditions needed for activation of cells to competence (31). These conditions include genetic competence (Table 2), de novo protein synthesis, and incorporation of choline. Table 3 also shows that electrophoretically pure activator could induce leakage and that leakage was not influenced by the addition of DNA. Cellular leakage measured by the release of other markers (hemolysin, autolysin, and exonuclease) gave similar results.

Transfer of the bacteria to the salt solution by various means did not significantly alter the release of enzymes. Collection of the activated bacteria by high-speed sedimentation, low-speed sedimentation onto a shelf of 60% sucrose,

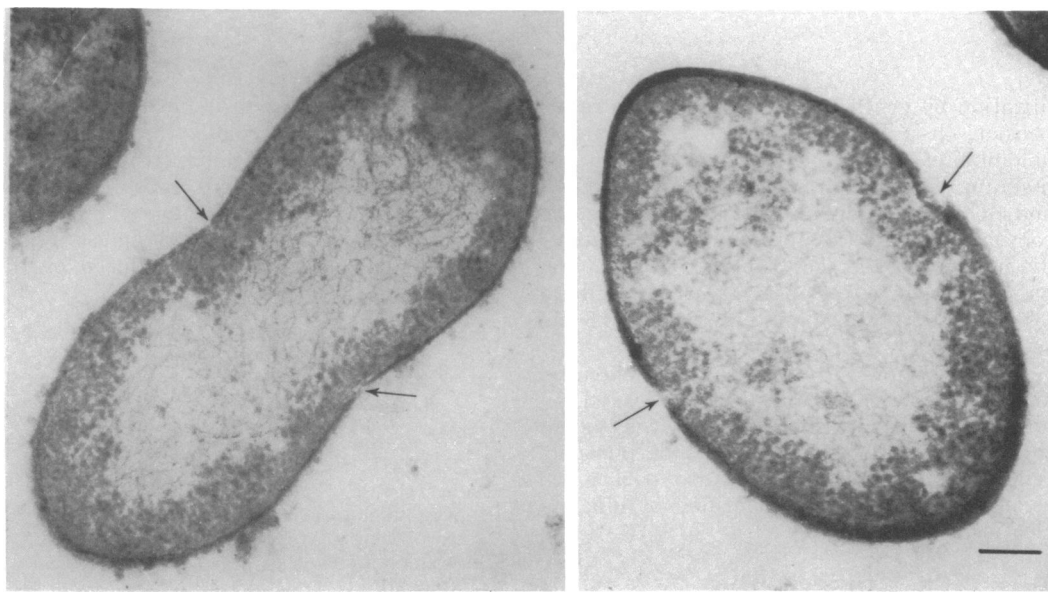

FIG. 3. *Localized cell wall damage in activator-treated pneumococci. Arrows indicate signs of limited autolytic activity. Magnification ×121,000. Bar 0.1 μm.*

TABLE 2. *Activator-dependent leakage of intracellular components to the medium*

Strain	Activator	Transformants (per ml)	β-galactosidase[a] (OD_{410})	Hemolysin (OD_{641})	Nuclease (counts/min)	Autolysin (counts/min)	[^{3}H]uracil (counts/min)	[^{3}H]phenylalanine (counts/min)
R6 (wild type)	+	3×10^6	0.34 (3-5%)	0.63 (10-14%)	12,400 (3-9%)	2,000 (4-5%)	67,000 (7-9%)	408 (5%)
	−	<300	0.06	0.00	1,600	0	22,000	81
RA7 (genetically incompetent)	+	<300	0.012 (<0.2%)	0.100 (2%)	0	0		
	−	<300	0.030	0.090	0	0		
cwl⁻ (autolysin transformant of hex)	+	1.1×10^6	0.00	0.01	3,000 (1.2%)	0	38,000 (5%)	
	−	<300	0.00	0.02	1,200	0	7,900	
hex	+	4×10^6	0.33 (5%)	0.75 (14%)	1,600 (5%)	1,800 (5%)		
	−	<300	0.06	0.01	1,300	0		

TABLE 3. *Effect of experimental conditions on cellular leakage*

Strain	Experimental conditions in phase 2	Transformants (per ml)	β-Galactosidase released (OD_{410})
R6 (cells grown in choline medium)	pH 8, no activator	<300	0.076
	pH 8, +activator	5×10^6	0.256
	pH 8, +activator + competing DNA	9×10^3	0.266
	pH 8, +electrophoretically homogeneous activator	10^6	0.375
	pH 6.6, +activator	400	0.056
	pH 8, +activator + chloramphenicol (100 μg/ml)	<300	0.075
R6 (cells grown in ethanolamine medium)	pH 8, + choline, no activator	<300	0.051
	pH 8, + choline, +activator	10^6	0.200
	pH 8, no choline, +activator	<300	0.060

filtration by gentle vacuum, or vigorous resuspension on a Vortex mixer gave comparable amounts of leaked enzyme activities in the medium. Furthermore, the presence of DNA and chloramphenicol and the lack of glucose (in combination with the inhibitor 2-deoxyglucose) did not inhibit leakage (Table 4). On the other hand, no leakage was observed at 0 C. Cellular leakage has a pH optimum between 7 and 8 and seems to follow a time course (Fig. 4, 5).

Table 5 shows the results of a chemical analysis of radioactive components leaked out of cells prelabeled with [^{3}H]uracil. It is evident that the leaked components include phosphorylated components of the precursor pool as well as material tentatively identified as oligonucleotides.

DISCUSSION

For the induction of cellular leakage and protoplast formation, two sets of conditions are required. First, the bacteria have to be treated with activator under exactly the same conditions that are needed to induce the competent state. Next, the competent cells have to be transferred to certain postincubation media.

Role of activation. Both protoplast formation and leakage seem to be induced by the

TABLE 4. *Effect of experimental conditions (phase 3) on cellular leakage*

Conditions in phase 3	β-Galactosidase released (OD_{410})[a]
Usual (SPA)	0.248
SPA at 0 C	0.040
SPA without glucose, +2-deoxyglucose (2 mg/ml)	0.203
SPA + chloramphenicol (100 μg/ml)	0.245
SPA + DNA (20 μg/ml)	0.228

[a] Optical density at 410 nm.

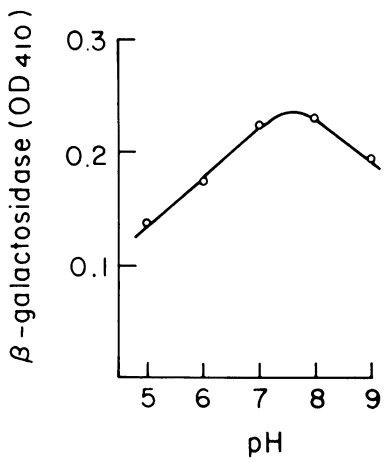

FIG. 4. *pH dependence of β-galactosidase leakage. Activator-treated bacteria (strain R36A) were suspended in SPA solution buffered at the pH indicated, and β-galactosidase activity was assayed in the supernatants.*

same substance that induces competence. This conclusion is based on two findings. First, activator purified to electrophoretic homogeneity can induce not only competence, but also leakage and protoplast formation. Second, leakage and protoplast formation only occur if the activator treatment is performed under the conditions essential for the induction of competence. A number of conditions that allow cellular binding of the activator but no competence (e.g., treatment with the activator at low pH, ethanolamine-containing media, the presence of chloramphenicol) cause no leakage or protoplast formation. Similarly, negative results were obtained with a genetically incompetent strain that can bind the activator but that does not undergo subsequent conversion to competence.

Postincubation conditions. Neither competent nor incompetent pneumococci show leakage or protoplast formation if the bacteria are kept in full growth medium. For detectable leakage and protoplast formation, the competent bacteria have to be exposed to a set of postincubation conditions. In the case of protoplast formation, this condition appears to be, in essence, a nutritional and temperature "shift-up" and high concentration of sucrose. Leakage requires brief incubation in buffered salt solution containing divalent cations, glucose, and albumin. It is important to note that the effects of these postincubations are not absolutely specific for the competent (activated) cells. While activator-treated pneumococci form protoplasts in 10 to 30 min, even the incompetent bacteria will start gradually converting to protoplasts upon prolonged (2 h) incubation in the high-sucrose medium. Similarly, leakage of enzymes reaches a maximum rapidly (within 10 to 15 min) in competent cells; the incompetent cells will become leaky after 30 to 40 min in the

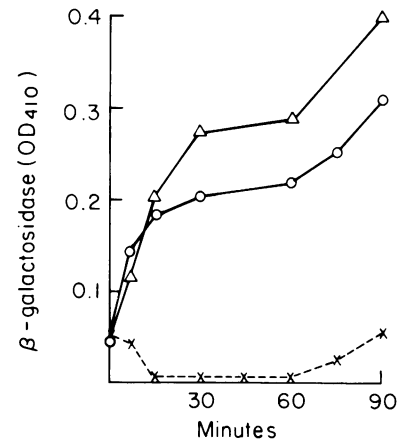

FIG. 5. *Time course of β-galactosidase leakage. Activator-treated (solid lines) and untreated (dashed line) pneumococci were incubated in SPA solution for the time periods indicated, and the β-galactosidase leaked to the medium was determined. Triangles and circles represent the results of two separate experiments.*

TABLE 5. *Analysis of radioactive uracil-labeled compounds that escaped into the medium*[a]

Total counts/min released	Total counts/min released (%)			TCA-soluble fraction	
	Alkali sensitive; TCA insoluble	Alkali resistant; TCA insoluble	TCA soluble	Type	Counts/min (%)
67,000 (7-9% of total cellular radioactivity)	29	2	69	Oligonucleotide-like	17
				Nucleoside polyphosphates	26
				Nucleoside monophosphates and UDP-X[b]	30
				Nucleoside and base	37

[a] TCA, Trichloroacetic acid.
[b] UDP-X, Uridine diphospho sugars, hexosamines.

leakage medium. In short, it seems that neither competent nor incompetent pneumococci are completely stable under the postincubation conditions and that the activator treatment greatly exaggerates this instability.

These observations suggest that the activatr treatment introduces a subtle damage or alteration into the cellular permeability barrier that may be amplified by the postincubation conditions. In full growth medium (i.e., in the absence of postincubation), the activator-induced "damage" may be rapidly repaired.

Mechanism. The fact that practically all cells in the activator-treated cultures can rapidly convert to protoplasts indicates that protoplast formation (and leakage) are properties of each individual competent cell. An alternative explanation would be that the escape of 1 to 5% of various intracellular enzymes to the medium represents complete lysis of a corresponding small fraction of the population. The autolysin liberated by these lysed cells would then attack the cell walls of the rest of the bacteria from without and cause protoplast formation. This is unlikely, however. Microscope observations and viability determinations show no signs of lethality of the bacteria in the leakage medium. Furthermore, experience with protoplast formation in pneumococci shows that the amount of autolysin leaked into the medium is several thousand times less than the concentration needed to induce protoplast formation. Similarly, if leakage represented the complete lysis of a small (5 to 10%) fraction of the population, one would expect the various leaked cell components to appear in the medium in the same relative proportions of their total cellular concentration. This was not the case (Table 2).

Thus, it seems that protoplast formation is a secondary consequence of the activator-induced leakage of autolysin molecules. These enzyme molecules, on route to the medium, could cause sufficient damage to the cell wall from within to allow emergence of protoplasts (autoplasts).

Physiology of leakage. Several observations suggest that leakage is not simply the consequence of the greater mechanical fragility of competent cells but, rather, it may involve an active process. (i) Leakage has a time course. (ii) Collection of activator-treated cells by "gentle" versus "drastic" means does not seem to influence leakage (or protoplast formation). (iii) Leakage does not occur at low temperature and it has a pH optimum.

The physiological requirements of leakage are distinct from those of DNA uptake since glucose-deprivation, removal of divalent cations, and addition of DNA have no effect on leakage (23).

Competent cells rapidly leak 1 to 5% of a variety of intracellular enzymes into the medium. Upon further incubation, one can detect a second wave of more extensive leakage, the time-course of which roughly parallels the leakage of incompetent cells.

The nature of the components that escape into the medium suggest a primary damage to the plasma membrane and not to the cell wall. This is suggested by the appearance of typical intramembrane markers (β-galactosidase and nucleoside phosphates) in the medium. Interestingly, activation of the autolysin-defective mutant to high levels of competence was accompanied by leakage of nucleoside phosphates, but not by macromolecular (enzyme) markers. It seems that both wild-type and mutant cells undergo the common primary (membrane) damage; on the other hand, the appearance of macromolecular components (enzymes) in the medium requires the additional loosening of the second permeability barrier (cell wall) by a sufficient number of autolysin molecules leaked onto the interior surface of the cell wall. In the autolysin-defective mutant, the macromolecular cell components may leak into the periplasmic space between wall and membrane and would be retained there owing to the very low concentration of autolysin. The low-molecular-weight nucleoside phosphates may find their way to the outside medium through the porosity of the cell wall.

Cellular leakage and the competent state. What conclusions can one draw concerning the competent state and the mechanism of action of the activator from the observations described in this paper?

The observations clearly indicate an alteration (or subtle damage) to the pneumococcal plasma membrane in the competent state. Under appropriate conditions, this membrane damage can be exaggerated to either protoplast formation or extensive leakiness. Since activated cultures in full growth media can undergo genetic transformation without any detectable leakage (30) or protoplast formation, one must consider the possibility that these two novel and dramatic phenomena are simply nonspecific consequences of membrane destabilization. Although this interpretation cannot be ruled out, it is not easy to reconcile with several features of our findings, namely, with the specific requirement for competence-inducing conditions and factors and with the physiological features of leakage.

A more attractive alternative explanation of our findings would be that the postincubation conditions only amplify, to detectable levels, membrane leakage and internal cell wall dam-

age, both of which may be essential correlates of the induction of competent state but which occur only at extremely low levels.

Such an interpretation would be fully compatible with one of the two specific models that have been proposed for the mechanism of competence (32). It would seem worthwhile to spell out details of this "unmasking" model, extended by the new findings reported here. The competent condition would be initiated by a membrane alteration resulting from the interaction between activator molecules and receptors on the plasma membrane (39), possibly at a special area (e.g., at mesosomal junctions under the cell wall growth zone [36]). The result of this interaction would be a limited leakage (possibly from a mesosome) of autolysin (and other enzymes) into the periplasmic space. Next, a limited action of these autolysin molecules from within would loosen up the cell wall structure sufficiently to unmask a DNA-binding protein component(s) of the plasma membrane that is present both in competent and incompetent bacteria. Recently, we suggested that such a DNA-binding protein might be a membrane-bound nuclease or some other enzyme(s) that has a normal role in the DNA metabolism of the cell (23).

A direct interaction of activator and the plasma membrane is compatible with the known localization of the "receptor" protein (39) and also with some of the unusual properties of activator, namely, its tendency for selfaggregation and stickiness to surfaces (35). In the model outlined above, the role of activator would be that of a highly specialized permeability-causing agent. In all recent theories about the physiological role of autolytic enzymes, these enzymes are assumed to have at least a temporary access to the cell wall at the growth zone (21, 24, 37). This, in turn, requires a transport of autolysin molecules from their sites of synthesis (ribosomes) to the outer surface of the plasma membrane. One is tempted to speculate further and suggest that proteins structurally similar to the activator, or the activator itself (at low concentrations or in modified form), may be part of this autolysin transport mechanism. The phenomenon of competence might then be the result of a relatively minor modification of this system (e.g., overproduction and/or secretion of the activator). Even a slightly elevated level of autolysin molecules in the periplasmic space would cause localized hydrolysis of bonds in the cell wall structure, somewhat in excess of what might be needed for normal growth and cell division.

In this model, the chloramphenicol-sensitive process (29) that is known to follow the activator's attachment to the cells would also be part of the unmasking mechanism. The requirement for new protein synthesis may represent either the synthesis of new autolysin molecules or may have to do with their transport or activation at their sites of action.

This postulated need for a class of autolysin molecules is closely analogous to the interpretation of the requirement for protein synthesis in antibiotic-induced lytic phenomena (20, 25, 33).

The unique feature of the unmasking model for competence is that it minimizes the number of cell components, the sole function of which would be to catalyze a step in genetic transformation. Instead, the cellular binding of DNA molecules would exploit normal physiological processes such as the activity of autolysin molecules at the surface and the formation of enzyme-substrate complexes between structure-bound DNA-specific enzymes and extracellular DNA molecules.

The model described includes two key assumptions. The first assumption is that the activator can directly cause permeability change in the plasma membrane. The validity of this assumption, at least in its extreme case, could be tested by using a membrane vesicle system (8). Second, the model for competence assumes the need for a limited cell wall damage (i) by autolysin molecules acting from within (ii) at the cell wall growth zone (iii); furthermore, this wall damage would be the consequence of the accelerated transport (or leakage) of autolysin molecules through the plasma membrane (iv). Although each one of these points (i through iv) are hypothetical, they are compatible with a large number of indirect observations in the literature. Partial exposure of the plasma membrane during the competent state and a possible role of autolysins in the phenomenon of DNA uptake have been repeatedly suggested for several bacterial transformation systems (2, 5, 19, 26, 27, 38). Furthermore, the cell wall growth zone seems to be involved with DNA binding of competent pneumococci (36). Recently, we presented evidence suggesting that the activator triggers a process of unmasking preexisting DNA binding sites at a deeper layer of the plasma membrane (23). Consistent with this notion, it was found that at least 50% of the DNA bound to competent pneumococci in a deoxyribonuclease-sensitive form is attached to sites on the plasma membrane (H. Seto and A. Tomasz, unpublished data).

In an apparent contradiction to the postulated role of autolysins in competence, Lacks (10) isolated a mutant resistant to deoxycholate-induced autolysis and yet capable of devel-

oping normal levels of competence. However, careful analysis of this mutant showed that it does contain a low, but demonstrable, level of autolysin. For the limited cell wall digestion invoked in the model, the availability of even a small complement of autolysin molecules would be sufficient. A true testing of the validity of this assumption would require the isolation of mutants with temperature-sensitive autolysin.

The observation described here may be related to the liberation of biologically active DNA molecules into the medium of growing pneumococcal cultures (16). The largest quantity of DNA in the filtrates was found when the cultures passed through their spontaneous competent phases. Since in wild-type pneumococci the competent phase is induced by an endogenous activator, it is possible that in this spontaneous system DNA secretion into the medium is registering the same kind of activator-induced leakage as described in this paper.

Recently, Lacks (11) described the isolation of a mutant that lost two features of competence development typical in the wild type, namely, the trypsin sensitivity and cell concentration dependence of competence. Yet, interestingly, the mutant was still producing a trypsin-sensitive activator and still retained the chloramphenicol-sensitive step in the expression of competence. It may be that in this mutant the activator performs its usual role internally rather than in the typical extracellular fashion. It would be interesting to test how these mutant cells would behave in the physiological tests of membrane leakage and protoplast formation.

Extensive nucleolytic degradation of DNA molecules adsorbed to the surface of competent bacteria has been noted in several laboratories (4, 11, 13, 23). The possible presence of nucleases in the periplasmic space and in the medium of competent bacteria will have to be taken into consideration in the interpretation of the physiological significance of such enzyme activities.

Several investigators have described a variety of biological and enzymatic activities in impure preparations of "competence factors" (1, 9, 17, 22). At least one of such activities (causing loss of cellular viability) was shown to be attributable to a contaminating bacteriocin (22). Since competence factors are prepared from competent bacteria or their culture filtrates, the possibility of leakage of a variety of cell components by such cells should further underline the caution one must exercise before interpreting activities of such preparations.

By analogy, other transformable bacteria may also be leaky in the competent state. Fragility of competent cells has been noted in several transformation systems, and the presence of colicins, nucleases, and DNA in the culture medium of such cells may be indicative of cellular leakage. It seems worthwhile to consider the possibility that the general biosynthetic latency of competent *Bacillus subtilis* (12) and the unusually long lag time in the expression of newly introduced genetic traits (15) may be caused by loss of intracellular precursor pools and membrane damage that might be less easily repairable than the one in pneumococci.

The phenomena described in this paper provide a glimpse into the fragile and intricate organization of the pneumococcal cell surface and into the mechanism of the complex physiological events that even minor perturbations of the cell surface structures can provoke. Such pleiomorphy has been frequently observed in various cell surface phenomena.

Finally, it may be of interest to point out that a temporary leakage of intracellular components during phage infection has been observed (18). The cellular leakiness described in this paper provides still another analogy between the physiology of virus infection and transformation (32).

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