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Relationship Between the Competence Antigen and the Competence-Activator Substance in Pneumococci

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ABSTRACT

TOMASZ, ALEXANDER (The Rockefeller Institute, New York, N.Y.), AND SAMUEL M. BEISER. Relationship between the competence antigen and the competence-activator substance in pneumococci. J. Bacteriol. **90**:1226–1232. 1965.—Antisera prepared against pneumococci in their competent phase inhibit deoxyribonucleic acid (DNA)-mediated genetic transformation as well as binding of radioactive DNA by the cells. The same sera do not inhibit transformation of competent *Haemophilus influenzae* and *Bacillus subtilis* cells, but transformation of a *Streptococcus* strain genetically related to pneumococci is inhibited. The kinetics of immune inhibition of transformation resembles the inactivation of bacteriophage by phage-neutralizing antisera. The appearance of the competence antigen on the surface of pneumococci can be induced by the competence-activator substance. Antisera prepared against competent pneumococci can also inhibit the conversion of incompetent cells to competence by the competence-activator substance. The possibility is considered that part of the new antigenic determinant appearing on the cell surface during competence may be the activator itself.

During their competent phase, pneumococcal cells possess a new antigenic determinant which is absent from the same cells in the incompetent physiological state (Nava, Galis, and Beiser, 1963). Antisera prepared against competent cells by injecting rabbits with formalin-killed pneumococcal cells could inhibit the transformation of competent pneumococci by deoxyribonucleic acid (DNA), whereas sera prepared against incompetent pneumococci had no inhibitory effect on the transformation.

The rabbit sera prepared against both the competent and the incompetent cells contained high titers of antibodies against the common pneumococcal surface antigens; however, sera prepared against competent cells contained, in addition, a minor component which could inhibit the transformation of competent pneumococci. The nature of this inhibitory effect suggested that the new antigenic determinant of the competent cells is a chemical structure which is part of the cellular site involved in the first stages of the transformation process.

Recent investigations have shown that the competent physiological state in pneumococci is "induced" and controlled by a proteinlike cell product—the activator (Tomasz and Hotchkiss, 1964)—which seems to be localized on the cell surface (Tomasz, *in preparation*). It appeared of substantial interest to investigate the relationship between the competence antigen and the pneumococcal activator.

MATERIALS AND METHODS

The bacterial cultures used in this work were all derivatives of the Avery R36A strain—a noncapsulated variant of pneumococcus type II (McCarty, Taylor, and Avery, 1946), subline R6. The methods used for the preparation of DNA (Marmur, 1961), and for growing of the competent and incompetent cells, and the procedures used for testing the competence of the cells, have been described (Tomasz and Hotchkiss, 1964). The activation of incompetent cells to competence was terminated by the addition of 0.1 μg/ml of subtilisin (Nagarse; Teikoku Chemicals, Osaka, Japan). Competent and incompetent cells were stored by freezing the cultures at a concentration of 5×10^7 cells per milliliter in medium containing 10% glycerol (Fox and Hotchkiss, 1957). Such frozen-cell preparations were melted in an ice bath and then incubated, after dilution or transfer to fresh medium by Millipore filtration, at 30 C for 10 to 15 min prior to use. This "resuscitation" after storage in the frozen state was found essential for obtaining reproducible results.

Assaying of the inhibitory titer of antisera was routinely done by incubating antisera diluted in

saline with 5×10^6 to 1×10^7 highly competent cells at 30 C for 30 min; the antiserum was diluted to ineffective levels, and a saturating concentration of DNA carrying the streptomycin-resistance marker was added to the cell suspension. After 20 min of incubation at 30 C, the transformation process was terminated by the addition of pancreatic deoxyribonuclease (twice recrystallized; Worthington Biochemical Corp., Freehold, N.J.), and the number of transformants was determined according to the usual procedure (Hotchkiss, 1954).

The γ-globulin nature of the inhibitory serum protein was tested by reacting samples of rabbit anti-competent pneumococcus sera (anti-co$^+$ sera) with excess goat anti-rabbit γ-globulin sera (kindly supplied by Curtis A. Williams, Jr.). As control for a possible inhibitory effect of the goat serum itself, rabbit sera prepared against incompetent pneumococcus (anti-co$^-$ sera) were also treated with goat serum. After incubation of the mixtures at 37 C for 1 hr followed by overnight in the refrigerator, the precipitated rabbit γ-globulin was removed by centrifugation, and the titer of the inhibitory component was determined in the supernatant fractions in the usual way.

In testing the interaction of antisera with DNA, 0.1-ml samples of undiluted antiserum were mixed with a series of concentrations of DNA in test tubes containing growth medium. After 30 min of incubation at 30 C, competent cells were added, and the inhibitory titer of the antiserum was determined. Cultures of α-hemolytic *Streptococcus* strain D [the strain used by Bracco et al. (1957), kindly supplied by M. Krauss of C. M. McLeod's laboratory] were grown in C-medium supplemented with fresh yeast extract (Tomasz and Hotchkiss, 1964). Pneumococcal activator was prepared by the following procedure. Highly competent cells were suspended in saline buffered with potassium phosphate buffer (0.01 M, *p*H 7.6) and containing 2-mercaptoethanol, and were heated for 10 min at 60 C to kill the cells and solubilize the activator substance. The suspension was centrifuged in the cold at 10,000 $\times$ *g*, and the supernatant fraction was used. The method used for the preparation of antisera was previously described (Nava, Galis, and Beiser, 1963). Three separate preparations of anti-co$^+$ and anti-co$^-$ sera were used in the experiments. The sera prepared against the competent and incompetent pneumococci had the same titer with respect to antibodies against the common pneumococcal antigens. The antisera had no detectable effect on the viability of highly competent or incompetent cultures.

Radioactive DNA was prepared from a uracil-requiring pneumococcal mutant resistant to streptomycin, which had been grown in C-medium (Tomasz and Hotchkiss, 1964) to which 0.1 μc/ml of uracil-2-C^{14} (specific activity, 20 to 30 μc/mmole; New England Nuclear Corp., Boston, Mass.) and 5 μg/ml cold uracil had been added. The incorporation of radioactive DNA into competent cells during transformation was determined as follows.

TABLE 1. *Effect of pretreatment of the anti-co$^+$ sera with DNA*

DNA concn	No. of transformants per ml after preincubation	
	Without anti-co$^+$ serum	With anti-co$^+$ serum
μg/ml		
1.6	1.1×10^6	2.2×10^5
1.0	0.98×10^6	2.2×10^5
0.5	1.1×10^6	2.2×10^5
0.16	0.9×10^6	2.2×10^5

The incubation of the competent cells with C^{14}-DNA at 30 C was terminated by chilling the suspension on ice; after centrifugation in the cold, the cells were suspended in growth medium to which 10 μg/ml of deoxyribonuclease were added, and the suspension was incubated at 37 C for 10 min. After three washings with fresh medium, the cells were lysed by the addition of a few drops of 5% deoxycholate solution and were plated on planchets. Radioactivity was determined in a windowless gas-flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

RESULTS

Specificity of immune inhibition. The antibody nature of the inhibitory component of sera was indicated by two features of this material: (i) the substance(s) which inhibited the transformation process was in the γ-globulin fraction of the sera, and (ii) the production of this inhibitory component in rabbits depended on injection with the specific kind of cell. Sera taken from rabbits prior to injection with formalin-killed competent cells did not have any inhibitory effect on transformation.

The nature of the inhibitory serum component was further investigated in the three experiments summarized in Tables 1 and 2. Preincubation of the transforming DNA with a high concentration of antiserum did not affect the inhibitory titer of the serum (Table 1), indicating that the inhibition was not due to an interaction with DNA.

Short pretreatment with papain (1 μg/ml, 30 C, 15 min) destroyed the inhibitory component of the serum (Table 2). The data in Table 2 also show that a considerable fraction of the inhibitory component(s) of the serum could be removed by precipitation with goat anti-rabbit γ-globulin serum. The incomplete restoration of the uninhibited level of transformation was apparently due to the presence of some unspecified inhibitory component in the goat serum, since an identical lowered level of transformation was obtained in the control samples also (i.e., in the tubes with anti-co$^-$ sera mixed with goat anti-rabbit γ-globulin).

Table 3 shows the results of experiments on

TABLE 2. *Effect of pretreatments of the anti-co$^+$ sera with papain (experiment 1) and goat anti-rabbit γ-globulin serum (experiment 2)*

Expt	Pretreatment	No. of transformants per ml
1	No anti-co$^+$ serum added	2.5×10^4
	With anti-co$^+$ serum	5×10^3
	With papain-treated anti-co$^+$ serum	3×10^4
2	No antiserum added	7×10^4
	With anti-co$^+$ serum	30
	With anti-co$^-$ serum	7×10^4
	With anti-co$^+$ serum after removal of γ-globulin	1.5×10^3
	With anti-co$^-$ serum after removal of γ-globulin	1.2×10^3

TABLE 3. *Species specificity of the antiserum prepared against competent pneumococci*

Competent cells used	No. of transformants per ml		
	No antiserum	With anti-co$^+$ serum	With anti-co$^-$ serum
Haemophilus influenzae	8.8×10^5	1.0×10^6	1.1×10^6
α-Hemolytic *Streptococcus* strain D (of Bracco et al., 1957)	5.3×10^3	90	5.3×10^3

the species specificity of the immune inhibition. High concentrations of the anti-co$^+$ sera caused no inhibition of the transformation of competent *Haemophilus influenzae* cells. (These experiments were kindly performed by Grace Leidy, Columbia University.) On the other hand, the transformation of competent cells of a *Streptococcus* strain closely related to pneumococci, the α hemolytic *Streptococcus* strain D (Bracco et al., 1957), was inhibited by the anti-co$^+$ serum.

The anti-co$^+$ serum had no effect on the transformation of competent *Bacillus subtilis* 168-2 (indole$^-$, leucine$^-$). (This experiment was performed in collaboration with David Dubnau, Albert Einstein College of Medicine.)

A suspension of pneumococci activated to competence by cell-free activator (about 10^8 cells per milliliter) was distributed in 5-ml samples in three tubes; 0.1 ml of anti-co$^+$ serum was added to the cells in tube A, 0.1 ml of anti-co$^-$ serum was added to the cells in tube B; no antisera were added to tube C. After incubation at 30 C for 20 min, 0.1 ml of C^{14}-DNA was added to each tube; the number of transformants and the amount of radioactivity bound by the cells was determined after 20 min of incubation at 30 C according to the procedures described in Materials and Methods. Table 4 shows that the anti-co$^+$ serum inhibited the transformation as well as the binding of radioactive DNA.

Kinetic characteristics of the immune inhibition of transformation. Figure 1 shows the results of typical antibody titration experiments. The antiserum at various dilutions was allowed to react with 10^7 highly competent pneumococcal cells in a series of test tubes, and the inhibitory titer of the antiserum was determined according to the routine procedure. It can be seen that a dilution

TABLE 4. *Inhibition of the binding of radioactive DNA by anti-co$^+$ serum*

Preincubation of competent cells	No. of transformants per ml	DNA-C^{14} bound per 5 ml
		count/min
With anti-co$^+$ serum	4.3×10^5	100
Without antiserum	2.4×10^7	2,715
With anti-co$^-$ serum	2.8×10^7	3,200

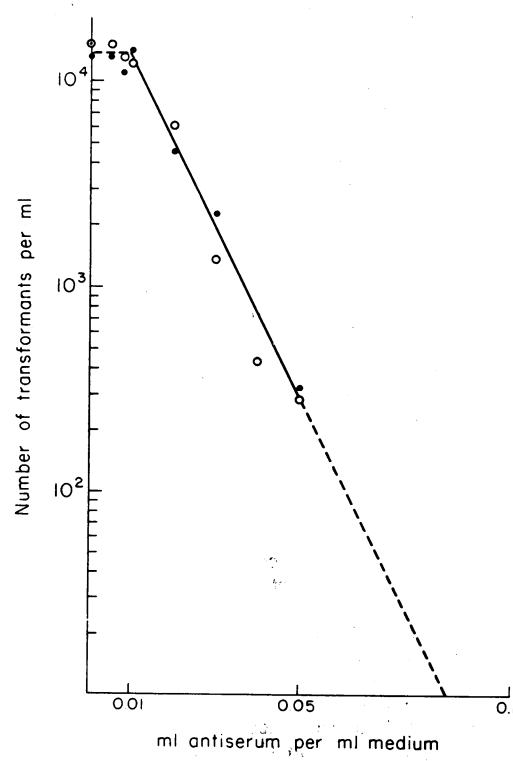

FIG. 1. *Titration of anti-co$^+$ serum with competent cells. Open and closed circles indicate two separate experiments.*

of the antiserum beyond 100-fold completely removed its inhibitory effect.

Figure 2 shows the kinetics of the inhibition. A series of dilutions of the antisera were added to test tubes containing identical concentrations of competent pneumococci, and the mixtures were incubated at 30 C for various times. (The inherent instability of the competent state limited the maximal duration of these incubations to less than 60 min.) Samples of the cells were then diluted 10-fold (to terminate the effect of antibody) into test tubes containing saturating concentrations of transforming DNA, and the number of transformants "surviving" the antibody inhibition was determined. It can be seen that the inactivation of the competent cells occurred by a first-order process. However, at higher dilutions of the antibody, the shape of the inactivation curves revealed a more complex process. The use of subsaturating DNA concentrations did not increase the effectiveness of the anti-co$^+$ serum (Fig. 3.)

By use of the data obtained with higher concentrations of antibody, where the inhibition appears to be a "single-hit" process, a K value of 4 or (in some experiments) a maximum of 6 to 8 was obtained, showing the relatively low titer of of the antibody preparations. (The K value is defined as the specific rate of immune inhibition:

$$K = 2.3\frac{D}{t}\log\frac{T_i}{T_t}$$

where T represents transformant, T_i indicates the number of transformants at zero-time, and T_t refers to the number of transformants at time t. D is the final dilution of the antiserum.)

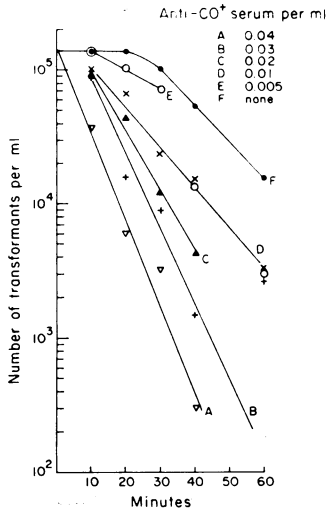

FIG. 2. *Rate of inactivation of competent cells by anti-co$^+$ serum.*

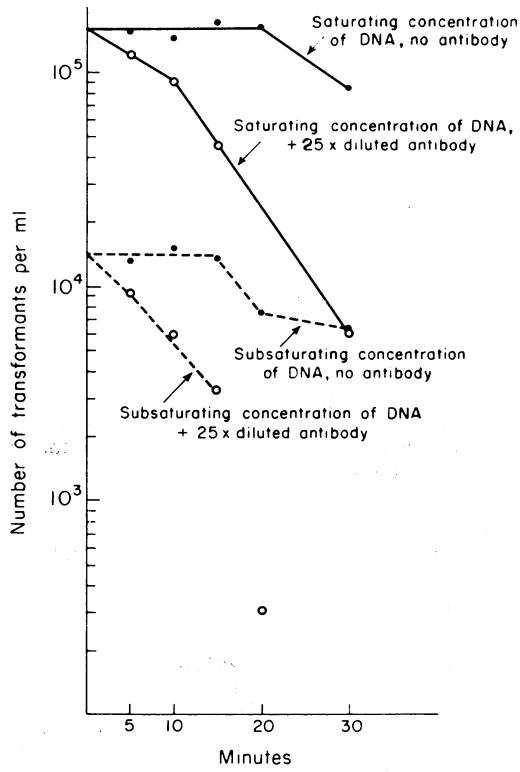

FIG. 3. *Rate of inactivation of competent cells by anti-co$^+$ serum. For the determination of the number of surviving transformants, saturating (0.2 μg/ml) and subsaturation (0.002 μg/ml) concentrations of transforming DNA were used.*

In subsequent experiments, the effect of varying the concentration of the competent cells on the extent of inhibition was determined. A minimal inhibitory concentration of antiserum was incubated with increasing concentrations of competent cells at 30 C for 30 min, and the extent of inhibition was determined by adding saturating concentrations of DNA and plotting the number of "surviving" transformants against the concentration of the competent cells. Figure 4 shows that the extent of inhibition was apparently independent of the concentration of the cells: at all cell concentrations used, a constant proportion of the cells was inhibited. This result is reminiscent of the behavior of phage-neutralizing antibodies (Burnet, Keogh, and Lush, 1947). This pesudo first-order kinetics presumably means that the number of antibody molecules per "competence site" is overwhelmingly high, even in the diluted sera and in the presence of high cell concentra-

TABLE 5. *Adsorption of the inhibitory activity of anti-co$^+$ serum with co$^+$ cells*

Treatment	No. of transformants per ml
No anti-co$^+$	5.6×10^5
Anti-co$^+$	2×10^2
Anti-co$^+$ adsorbed with co$^+$ cells	2×10^4
Anti-co$^+$ adsorbed with co$^-$ cells	6×10^2

tions, and that the number of competent sites per cell is probably not large.

The probability that the number of antibody molecules per "competence site" is very high also probably explains the difficulties encountered in attempts to adsorb the inhibitory activity from anti-co$^+$ sera with competent cells. Most adsorption attempts were unsuccessful, but, occasionally, with very heavy suspensions of formalinized co$^+$ and co$^-$ cells, and with repeated adsorption, evidence for a specific decrease in the inhibitory activity of anti-co$^+$ sera could be obtained (Table 5).

Immune inhibition of the transformation of cells activated to competence by purified activator preparations. A 2-ml suspension of incompetent pneumococci (10^7 cells per milliliter) was treated with 0.2 ml of cell-free activator preparation. After incubation at 30 C for 30 min, the activation of the cells was terminated by the addition of subtilisin (0.1 μg/ml). To a sample of the cell suspension, anti-co$^+$ serum was added (0.1 ml to 1 ml of suspension). The rate of inactivation of the competent cells is shown in Fig. 5. Figure 5 also shows the rate of inactivation of competent pneumococci which were allowed to develop competence "spontaneously" during growth. It can be seen that the kinetics of immune inhibition were similar with the two kinds of competent cells.

Inhibition of the activation process by anti-co$^+$ sera. The activation process in which incompetent cells are converted to competence as a result of the interaction with the activator is inhibited by low concentrations of proteolytic enzymes. The same enzymes have no effect on the transformation process, in which competent cells react with DNA molecules (Tomasz, *in preparation*). This different sensitivity to proteolytic enzymes provides an operational distinction between the two processes. Since, at the present time, at least, there is no way to assay the activation process independently of transformation, the effect of anti-co$^+$ serum on the activation process could only be determined in an indirect way.

Samples of an activator preparation of known activity were added to two test tubes, A and B, each containing equal concentrations of a standardized incompetent cell suspension. In addition, tube B also received anti-co$^+$ serum of known titer. Both tubes were incubated at 30 C for 30 min, at which time 0.1 μg/ml of subtilisin was added to all tubes to destroy the activator and to stop the activation process. (A control experiment showed that subtilisin had no effect on the inhibitory power of the anti-co$^+$ serum.)

Two 0.1-ml samples of tube A, A1 and A2, were diluted 10-fold into fresh media; both A1 and A2 received saturating concentrations of transforming DNA, and A2, in addition, received anti-co$^+$ serum in the same concentration as was used in tube B. A 0.1-ml sample from tube B was also diluted and treated with DNA. The three tubes were incubated at 30 C, and the transformation process was terminated 30 min later by the addition of deoxyribonuclease. The number of transformants was determined in the routine way (Table 6).

The number of competent cells in both tubes A1 and A2 must be the same; however, the actual number of transformants observed in tube A2 is less, since, in this tube, a fraction of the competent cells was prevented from reacting with DNA by the antiserum added with the DNA. The great decrease in the number of transformants in tube

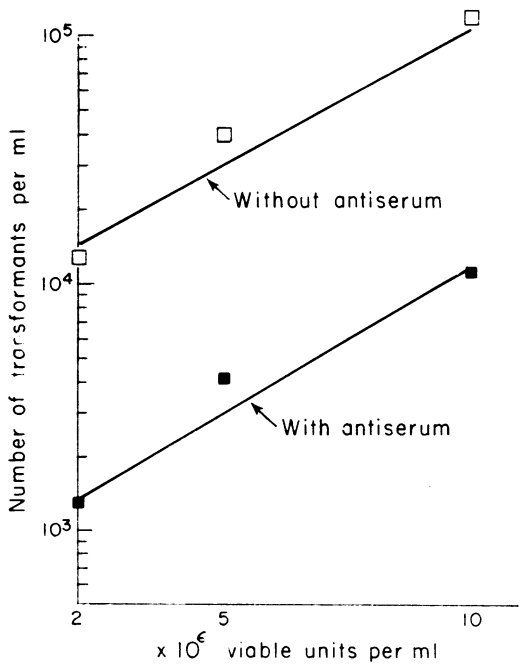

FIG. 4. *Effect of the concentration of competent cells on the extent of immune inhibition.*

B, as compared with tube A2, clearly reflects an inhibition of the activation process by the anti-co$^+$ serum.

Since antiserum prepared against incompetent cells had no effect on the activation process (see Table 6), the inhibition observed with the anti-co$^+$ serum seems to be caused by an interaction with the activator rather than by the interaction with some specific cellular site. This latter possibility was also made unlikely by experiments in which incompetent cells were allowed to react with anti-co$^+$ serum in the absence of activator for 30 min. The antiserum was subsequently removed by washing the cells on a Millipore filter. Upon resuspension and addition of activator, these cells could be converted to competence, showing at least that an irreversible binding of co$^+$ antibody by incompetent cells does not occur. A similar washing procedure performed with competent cells which have reacted with anti-co$^+$ serum did not cause reversal of the immune inhibition.

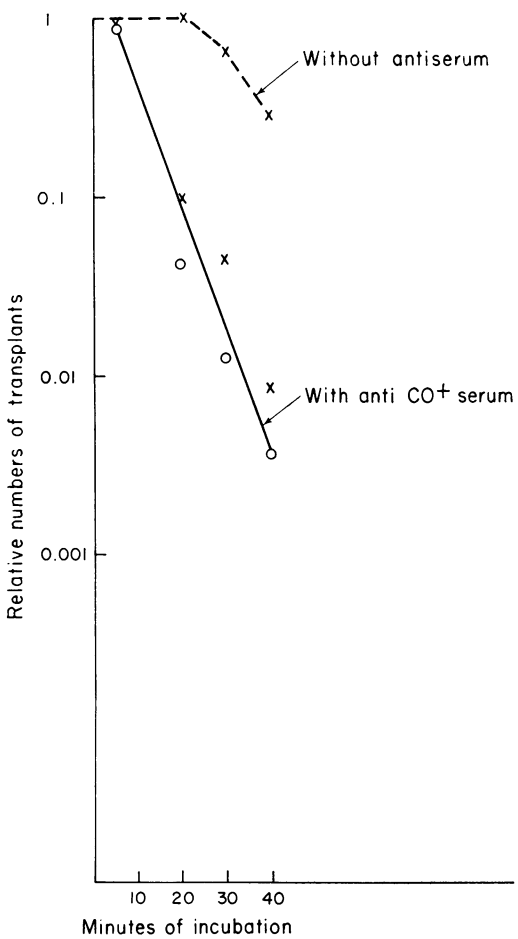

FIG. 5. *Rate of inactivation of competent cells by anti-co$^+$ serum. Symbols:* ×, *competent cells which developed the competent state spontaneously during growth;* ○, *cells activated to competence by cell-free activator preparation.*

TABLE 6. *Inhibition of the activation process by anti-co$^+$ serum*

Tube	Treatment	No. of transformants per ml
A1	No anti-co$^+$	1.5×10^5
A2	Anti-co$^+$ present during transformation	6×10^4
B	Anti-co$^+$ present during activation	3×10^2
	Anti-co$^-$ present during activation and transformation	1.5×10^5

An alternative explanation for the inhibition of the activation process by anti-co$^+$ serum would be that cells in the process of conversion to competence may go through a "precompetent" state in which they are much more sensitive to antibody inhibition than are "fully" competent cells (i.e., cells which have completed the process of conversion to competence).

DISCUSSION

The greatly decreased capacity of competent cells treated with anti-co$^+$ serum to bind radioactive DNA indicates that the mechanism of immune inhibition of the transformation process is an interference of the antibodies with the combination of DNA molecules with the cellular DNA binding sites. The antigenic structure of these sites seems to be specific for pneumococci, since pneumococcal anti-co$^+$ sera do not inhibit genetic transformation in other transformable species. The exception to this in the case of α-hemolytic *Streptococcus* strain D is particularly interesting, since the major pneumococcal surface antigen (R) seems to be absent from this streptococcus (Bracco et al., 1957). It is most reasonable to interpret the inhibition of genetic transformation in this streptococcus by antisera prepared against competent pneumococci as a further evidence for the close relation between pneumococci and this organism. Other evidence for a close taxonomic relation comes from successful interspecific transformation between the two species (Bracco et al., 1957) and from our recent finding that pneumococcal activator can activate the α hemolytic streptococcal cells, and, in turn, the activator of this streptococcal strain can activate pneumococ-

cus cells to competence (Tomasz, *unpublished data*).

The specific "competence antigen" on the surface of competent pneumococci appears to be produced in the interaction of incompetent cells and the activator. This conclusion may be inferred from the following findings: (i) incompetent cells do not possess the competence antigen (as judged from their inability to induce the production of anti-co$^+$ antibodies in rabbits) and (ii) such incompetent cells do develop this antigen during activation to the competent state by purified activator preparations (as judged by the fact that the transformation of competent cells produced in this way is inhibited by anti-co$^+$ sera).

The finding that competent cells produced by activation with cell-free activator preparations and cells which have developed the competent state during growth are inhibited with equal efficiency by anti-co$^+$ sera shows that the "competence sites" produced in the activation process are identical antigenically to those appearing on the cell surface during spontaneous development of competence. This is a further addition to the evidence (Tomasz and Hotchkiss, 1964; Tomasz, *in preparation*) suggesting that the spontaneous development of competence in growing cultures is the result of an activation process by endogenously produced activator. It is not known at present whether the appearance of the new antigenic sites during activation is a result of an "unmasking" process or involves de novo synthesis of the antigen.

The inhibition of the activation process by anti-co$^+$ sera raises the possibility that one of the new antigenic determinants on the competent cell surface may be the activator itself. Certain observations make this possibility a plausible one. It was shown earlier that the activator content of pneumococcal cultures closely parallels the level of competence (Tomasz and Hotchkiss, 1964). Furthermore, some recent experiments indicate that the activator is localized in the cell envelope, from which it can be solubilized as a trypsin-sensitive material of considerable molecular weight. The antigenic activity of such activator preparations is presently being tested.

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