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Inhibition of the Development of Competence in *Streptococcus sanguis* (Wicky) by Reagents that Interact with Sulfhydryl Groups: Discernment of the Competence Process

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Reagents that interact with sulfhydryl groups are shown to inhibit competence factor (CF)-induced competence development in *Streptococcus sanguis* (Wicky) strain WE4 (Wicky 4 Ery^R). Inhibition is correlated with specific inhibition of either the function or biosynthesis of three competent cell-related proteins and is reversed by either 2-mercaptoethanol or dithiothreitol. Mercuric chloride (5 μM) or *N*-ethylmaleimide (NEM; 50 μM) inhibited (i) the function but not the biosynthesis or activation of the competent cell-associated autolysin; (ii) the biosynthesis of a competent cell-associated protein of unknown function, demonstrated by polyacrylamide gel electrophoresis of acidified phenol extracts; and (iii) the biosynthesis or activation of distinct deoxyribonucleic acid (DNA)-binding sites. Neither reagent at the indicated concentration interfered with the uptake of CF by cells or with the uptake and expression of DNA by competent cells. Neither reagent inactivated CF or genetic markers coded by the transforming DNA, nor did they inhibit cell growth or viability appreciably. The data reveal that either mercuric chloride or NEM can differentially inhibit induced protein synthesis and, in addition, conclusively show that some autolytic activity is essential for the onset of the competent state.

A requirement for an autolytic event to initiate the development of competence has been hypothesized but never conclusively shown (1, 9, 13, 19). Recent kinetic evidence obtained with group H streptococcal cells showed that their potential to autolyze and their ability to develop competence (measured by genetic transformation) occurred simultaneously and both events reached maximal values at the same time. Moreover, group H cells that can transform but do not have the potential to autolyze have not been found (9).

Since the maximal extent of autolysis (greater than 90% decrease in optical density [OD]) requires the presence of a reducing agent (2-mercaptoethanol [2-ME] or dithiothreitol), the need for a free sulfhydryl group for activity is apparent. Sulfhydryl-reacting chemicals (mercuric chloride, *N*-ethylmaleimide [NEM], iodoacetate, and *p*-hydroxymercuribenzoate) were tested to determine whether inhibition of autolytic activity resulted in the inhibition of the development of competence.

The data presented in this report show that: (i) a very early autolytic event is essential for the development of competence, (ii) this autolytic event alone does not render a cell competent, and (iii) a minimum of three proteins, including the autolysin, are induced by competence factor (CF) and are required for the attainment of the competent state. The other two proteins are: (i) the one demonstrated earlier by polyacrylamide gel electrophoresis (PAGE) of acidified phenol extracts of competent cells (14) and (ii) a protein involved with deoxyribonucleic acid (DNA) binding (5).

MATERIALS AND METHODS

Organism. The organism used in this report is an erythromycin-resistant mutant of *Streptococcus sanguis* (Wicky), designated Wicky 4 Ery^R and abbreviated herein as WE4. It was obtained by transforming Wicky cells with DNA derived from an erythromycin-, streptomycin-, and novobiocin-resistant mutant of *S. sanguis* (Challis). The resulting strain (WE4) is resistant to 1.5 μg of erythromycin (ilotycin gluceptate, Lilly and Co., Indianapolis, Ind.) per ml and sensitive to the other antibiotics. It can be made 100% competent (9) by treatment with saturating amounts of CF derived from strain Challis and can yield high levels of transformants (10).

CF production. The CF-containing solutions used in these studies were derived from the supernatant

fluids of strain Challis cells grown for 3 h at 37 C in brain heart infusion (Difco) supplemented with 1% (wt/vol) neopeptone (Difco) and 2.5% (vol/vol) heat-inactivated horse serum (56 C, 30 min; Microbiological Associates, Inc., Bethesda, Md.). (For details see reference 13.)

Autolysis. Autolysis was measured as a decrease in OD at 750 nm by using a Beckman model B spectrophotometer.

Growth and maintenance of cultures. WE4 cells were grown at 37 C in brain heart infusion supplemented with 2.5% (vol/vol) heat-inactivated horse serum (BHS broth) and maintained at −40 C in BHS broth supplemented with 15% (vol/vol) glycerol.

Competence induction and transformation. WE4 cells were grown overnight at 37 C in BHS broth supplemented with 1.5 μg of erythromycin per ml (phase 1). These cells were then diluted to 5% (vol/vol) into fresh BHS broth and incubated statically at 37 C for 50 min (phase 2). At this time, heated CF-containing solutions (56 C, 30 min; 9, 11) were added for an additional 50 min (phase 3). At the end of phase 3, cells (usually 1.0 ml) were added to a DNA solution (0.1 ml) for a total of 30 min (phase 4). These cells were then diluted into 0.4% BH broth and plated in BH agar (phase 5). After 3 h at 37 C, the cells were overlaid with BH agar containing twice the final antibiotic concentration (dihydrostreptomycin sulfate, 500 μg/ml) and incubated for 48 h at 37 C (phase 6), after which time the colonies were counted. In all experiments, total colony-forming units were also determined. Any modification of this protocol will be stated.

Transforming DNA. Transforming DNA was prepared from autolyzed WE4 cells by a method already described (9).

Radioactive DNA. Radioactive DNA was prepared from autolyzed WE4 cells by a method already described (8, 9), except that the cells were grown in a semisynthetic medium (8) containing 1.0% (wt/vol) glucose, 2.5% (vol/vol) horse serum, and 2.5 to 5 μCi of [*methyl*-^{3}H]thymidine per ml (New England Nuclear Corp., Boston, Mass.; 6.7 Ci/mmol).

Chemicals. All chemicals were purchased from commercial sources.

Quantitative PAGE. PAGE was performed as described (14). The gels were stained with amido black (Canalco, Rockville, Md.; 0.5% [wt/vol] in 7 to 10% [vol/vol] acetic acid) for 60 min and destained electrophoretically (15 to 17 V/gel) and by diffusion in 7 to 10% (vol/vol) acetic acid. Tracings of the gels were made with a Photovolt Densicord 542 A densitometer using a red filter. The tracings were transferred from chart paper to Xerox 1524 dual-purpose white paper (75.2 g/m^2) by using a Xerox copier, and the peaks were excised with a scalpel. These were then weighed on an analytical balance. The data are expressed as a percentage of the total weight. There was no advantage in staining gels with fast green (6).

Binding of radioactive DNA to "competent" cells. Competent cells (1 to 2 ml) were treated with 0.05 ml of [H^3]DNA (about 6,000 counts/min) as each individual experiment required. After treatment (usually for 30 min at 37 C), the cells were harvested by centrifugation in an Eppendorf 3200 centrifuge, washed once with 0.85% (wt/vol) saline, and suspended in a small volume of saline. These cells were quantitatively transferred to glass scintillation vials, and radioactivity was counted with a Packard spectrometer (model 3002) using Aquasol (New England Nuclear Corp., Boston, Mass.) as the scintillator.

RESULTS

The sulfhydryl-binding reagents used in this study were NEM and mercuric chloride. These were used at minimal inhibitory concentrations of 50 and 5 μM, respectively. At these concentrations, NEM and mercuric chloride inactivated neither CF nor transforming DNA; they did not interfere with the uptake of CF by cells nor did they prevent transformation when added to fully competent cells. Other reagents used for the same purpose but to a limited extent were iodoacetate and *p*-hydroxymercuribenzoate.

Kinetics of inhibition of the development of competence by either NEM or mercuric chloride. Competence (measured by genetic transformation) was inhibited by either NEM (Table 1) or mercuric chloride (Table 2). Maximal inhibition was obtained when either reagent was added to cells simultaneously with CF or 10 min after CF addition. Previous experiments showed that 10 min at 37 C is enough time for cells to take up or bind over 95% of the added CF (unpublished observation; 7). Inhibition became less when either reagent was added to cells that had reacted with CF for longer periods of time.

Reversal of NEM or mercuric chloride inhibition of competence by 2-ME. Twenty millimolar 2-ME, when added simultaneously with CF and either mercuric chloride or NEM, can relax the inhibition of competence (Table 3). This was determined to be the best concentration to use even though it inhibited competence development to some extent (60 to 80%); higher concentrations inhibited more, whereas lower concentrations were less influential in reversing inhibition (data not shown). Reversal of inhibition was time dependent (see below). 2-ME could be replaced by an equal concentration of dithiothreitol.

Inhibition of autolysis by NEM or mercuric chloride. The above data showed that the development of competence could be reversibly inhibited by sulfhydryl-binding reagents. Earlier work showed that only competent cells or potentially competent cells (those that are treated with CF but do not bind DNA; e.g., lysogenized cells) have the ability to autolyze when placed into a pH 9 buffer containing 2-ME

TABLE 1. *Kinetics of inhibition of the development of competence by NEM in WE4 cells*[a]

Time of NEM (50 μM) addition to cells in phase 3	Transformation[b] (%)	Relative inhibition
0 min with CF	7.4×10^{-4}	2,500
10 min after CF	5.5×10^{-4}	3,450
20 min after CF	1.5×10^{-2}	126
30 min after CF	1.7×10^{-2}	112
40 min after CF	5.0×10^{-2}	38
50 min after CF	6.7×10^{-1}	2.8
None with CF	1.9×10^{0}	1.0

[a] NEM, *N*-ethylmaleimide. WE4 cells were grown in phase 1 in BHS broth (Difco) at 37 C and diluted to 5% (vol/vol) into 100 ml of fresh BHS broth contained in a 250-ml Erlenmeyer flask. The cells were incubated for 50 min in phase 2 at 37 C, at which time competence factor (CF) was added (20%; vol/vol). A 10-ml volume of culture was then distributed into test tubes, at each time period, that contained 0.1 ml of 5 mM NEM. Incubation was continued for 50 min in phase 3 at 37 C, at which time 1.0 ml of cells was added to 0.1 ml of a standardized solution of DNA. Cells and DNA were incubated for 30 min in phase 4 at 37 C, diluted, and plated. After incubation of the cells for 3 h in phase 5 at 37 C, they were challenged with dihydrostreptomycin sulfate. Transformed colonies and total colony-forming units were scored after 48 h of incubation in phase 6.

[b] Percent transformation is equal to the number of transformed colonies times 100 divided by the number of colony-forming units.

(8, 13). Since 2-ME is required for maximal autolytic activity, the need for a free sulfhydryl group is indicated. Therefore, a test was made to determine whether the inhibition of the development of competence was due to specifically inhibiting autolytic activity.

An initial experiment showed that, although cells treated simultaneously with CF and either NEM or mercuric chloride at zero time in phase 3 transformed at reduced frequencies (Tables 1, 2, and 3), they autolyzed almost maximally when placed into the 2-ME-containing lysing buffer (13). Since 2-ME can reverse mercuric chloride- or NEM-inhibited enzyme reactions, it remained unknown whether the autolysin or its hypothetical activator was inhibited.

To show that the function of the autolysin was indeed inhibited, cells were treated by the method of C. L. Parsons (personal communication). Parsons showed that autolysis of competent or potentially competent cells could be achieved in broth if inhibitors of energy metabolism (sodium fluoride, NaF, or sodium arsenate) were added to them. However, for observation of this phenomenon, the cells must be grown in phases 1, 2, and 3 in BHS broth prepared with BH obtained from Baltimore Biological Laboratories, Baltimore, Md. The results showing that mercuric chloride does inhibit autolytic activity are presented in Fig. 1. Transformation data are also given and were obtained from cultures treated as described in the legend.

Figure 1a shows control cells (only CF during phase 3; transformation frequency [TF] = 1.9%). NaF stimulated autolytic activity which was manifested as a decrease in OD. Cells treated with water grew. Cells treated during phase 3 with CF and mercuric chloride (TF = 0.003%) did not lyse when NaF was added (Fig. 1b). Autolytic activity was inhibited by mercuric chloride. Upon addition of 2-ME, however, NaF-stimulated lysis occurred. Figures 1c to 1f are various controls. Cells treated with CF during phase 3 (TF = 1.3%) did not lyse when mercuric chloride and NaF were added to them at the end of phase 3 (Fig. 1c). The resident autolysin was again fully inhibited. Cells treated with CF during phase 3 (TF = 0.8%) and then with NaF and 2-ME at the end of phase 3 autolyzed at a rapid rate (Fig. 1d). Cells treated with just 2-ME lysed to a small extent (20%) and, although not shown, eventually recovered and continued to grow. Cells grown in the presence of 2-ME, mercuric chloride, and CF during phase 3 (TF = 0.4%) lysed regardless of the addition of NaF (Fig. 1e). Apparently, these cells could not recover from the extensive damage caused by the 2-ME-stimulated autolysin. In addition, cells treated with CF and just 2-ME during phase 3 responded in a similar manner (data not shown). Cells not treated with CF during phase 3 (noncompetent control culture; TF = 1.2×10^{-4}%) did not lyse upon addition of NaF (Fig. 1f). The same result was obtained with similar cultures that received 2-ME during phase 3. This last result emphasizes the fact that the autolysin is associated only with competent or potentially competent cells. Iden-

TABLE 2. *Kinetics of inhibition of the development of competence by $HgCl_2$ in WE4 cells*[a]

Time of $HgCl_2$ (5 μM) addition to cells in phase 3	Transformation (%)	Relative inhibition
0 min with CF	1.5×10^{-3}	1,200
10 min after CF	2.0×10^{-3}	900
20 min after CF	1.3×10^{-2}	138
30 min after CF	2.4×10^{-2}	75
40 min after CF	1.3×10^{-1}	14
50 min after CF	8.4×10^{-1}	2.1
None with CF	1.8×10^{0}	1.0

[a] See footnote *a* of Table 1 for details.

TABLE 3. *Reversal of NEM or $HgCl_2$ inhibition of competence development by 2-mercaptoethanol (2-ME)*[a]

Time of addition in phase 3			Transformation[b] (%)	Relative inhibition
NEM, 50 μM	$HgCl_2$, 5 μM	2-ME, 20 mM		
0 min with CF			5.8×10^{-5}	40,000
0 min with CF		0 min with CF	8.1×10^{-1}	2.8
None with CF		0 min with CF	4.6×10^{-1}	4.6
None with CF		None with CF	2.3×10^{0}	1.0
	0 min with CF		7.5×10^{-3}	320
	0 min with CF	0 min with CF	6.5×10^{-1}	3.7
	None with CF	0 min with CF	9.0×10^{-1}	2.7
	None with CF	None with CF	2.4×10^{0}	1.0

[a] WE4 cells were treated essentially as described in footnote *a* of Table 1. After phase 2, the cells were diluted into BHS broth (Difco) containing CF and either NEM or $HgCl_2$, with or without 2-ME. After 50 min, the cells were incubated in phases 4, 5, and 6.

[b] See footnote *a* of Table 1.

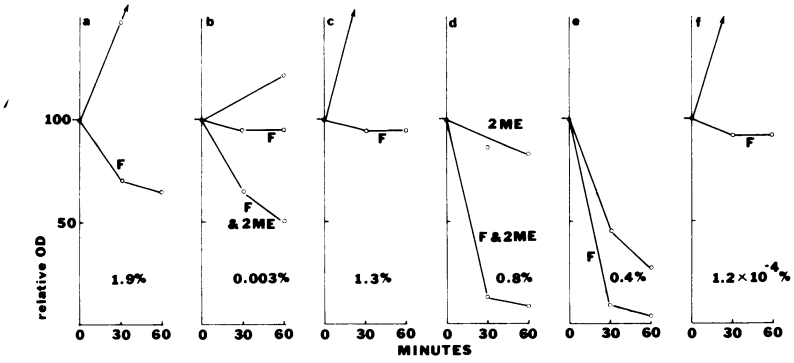

FIG. 1. *Inhibition of autolytic activity by mercuric chloride and its reversal by 2-ME. WE4 cells were grown in phases 1, 2, 3, and 4 in BHS (BBL) broth. Phase 3 treatments: (a, c, and d) cells were treated with 20% CF; (b) cells were treated with 20% CF and 5 μM mercuric chloride; (e) cells were treated with 20% CF, 5 μM mercuric chloride, and 20 mM 2-ME; (f) cells were treated with 20% BHS (Difco) in place of CF. After phase 3 incubation, a 1.0-ml sample from each culture was tested for transformation by using standard procedures. These data are expressed in the lower part of each figure as percentages. Treatment for autolysis: Control cultures in each Fig. received water (0.5 ml) in place of 0.5 ml of 1 M NaF (F); culture volumes were 5 ml. One culture received NaF and 20 mM 2-ME (b). (a, e, and f) No further additions after phase 3; (c) before addition of water or NaF, the cultures received 5 μM mercuric chloride; (d) before addition of water or NaF, the cultures received 5 μM mercuric chloride and 20 mM 2-ME. Data are recorded as the percentage of change in the starting optical density (OD) values. Incubations were done at 37 C.*

tical results (Fig. 1a-f) were obtained when NEM was used instead of mercuric chloride.

Kinetics of 2-ME reversal of mercuric chloride-inhibited competence development with or without the addition of CAP. The above results show that the reduction in the level of competence caused by mercuric chloride (or NEM; Tables 1 and 2) can be correlated with inhibition of autolytic activity (Fig. 1b) at an early time in phase 3 if one considers previous kinetic data which showed that autolytic activity can be observed 15 to 20 min after the addition of CF to WE4 cells (9). Additional evidence that supports the hypothesis that an autolytic event is essential for the development of competence was obtained by observing the kinetics of competence induction in cultures treated with mercuric chloride (or NEM).

The data shown in Fig. 2 were derived from cultures that were treated with CF and mercuric chloride and, at various times during phase 3, treated further with either 2-ME or 2-ME and chloramphenicol (CAP) 10 μg/ml, final concentration). CAP was added to prevent the synthesis of "new CF-induced" proteins. All cultures, including controls, were incubated in phase 4 with 10 μg of CAP per ml. When cultures were treated with CF and mercuric chloride, competence was fully restored when 2-ME was added during the first 10 min of incubation. (The control TF obtained with cultures treated with CF and 2-ME during phase 3 was 0.61%.) When

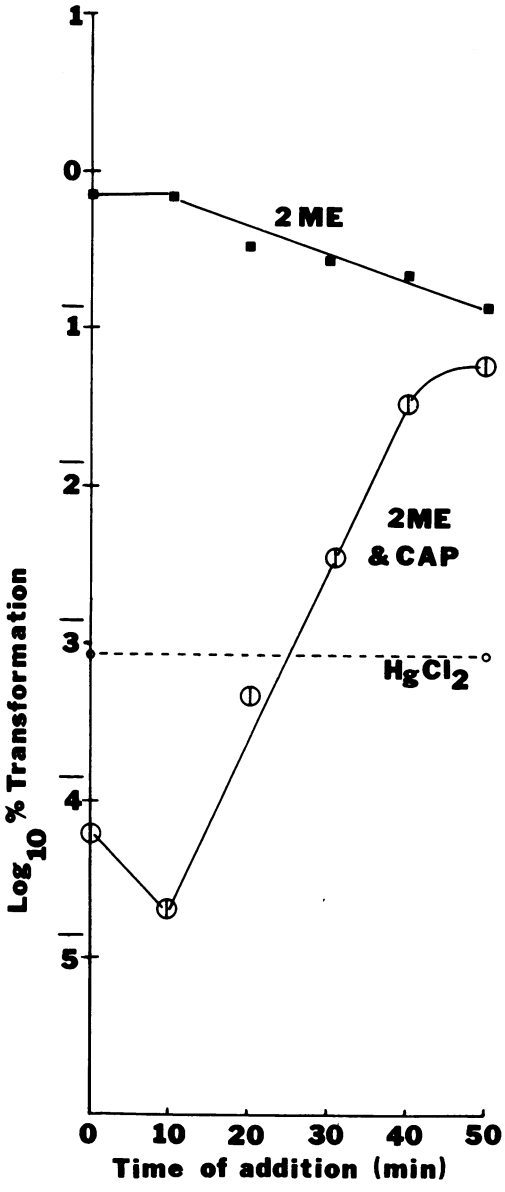

FIG. 2. *Kinetics of reversal of mercuric chloride inhibition of competence development by 2-ME in the presence or absence of chloramphenicol. WE4 cells were grown in phases 1 and 2 in BHS broth (Difco). At the end of phase 2, the cells were chilled in an ice bath and distributed into 15 test tubes. At zero time in phase 3, 5 μM mercuric chloride was added together with 20% (vol/vol) CF to 13 cultures; one culture received CF and 2-ME and another received just CF. These served as controls. The cultures were transferred to 37 C. Starting at time zero in phase 3 and at 10-min intervals thereafter, either 20 mM 2-ME or 20 mM 2-ME and 10 μg of chloramphenicol (CAP) per ml were added. At the end of phase 3, cells from each culture were added to a standard solution of DNA for 30 min (phase 4); the cells were treated further in phases 5 and 6. Cells that did not receive CAP during phase 3 were incubated with CAP (10 μg/ml) and DNA during phase 4. The dotted line shows the percentage of transformation in WE4 cultures that received mercuric chloride and CF during phase 3 with no other additions (mercuric chloride control). The 2-ME and CF control culture transformed to 0.61%; the CF control culture transformed to 1.5%.*

2-ME was added after 10 min, the level of competence decreased. This result shows that 2-ME reversal of mercuric chloride inhibition is time dependent.

When 2-ME was added with CAP, three important facts became apparent. The first was that the kinetic picture obtained was similar to that obtained with uninhibited cells (9, 10), except that the transformation frequencies were shifted downwards by about 25-fold. The second was that all mercuric chloride-inhibited cultures that received 2-ME and CAP transformed to lower frequencies than those cultures that received only 2-ME. The third and most important fact was that the level of transformation, and therefore competence, was maximally restored at the 50th min of phase 3 in cultures that received 2-ME and CAP at that time. The level of transformation reached was 66-fold higher than in mercuric chloride-inhibited cells. The only apparent change that occurred in this sample was the reversal of mercuric chloride inhibition of autolytic activity by 2-ME in the absence of additional protein synthesis.

In the same experiment, NaF-stimulated autolysis was observed in all cultures treated in phase 3 with 2-ME and only in those cultures that received 2-ME and CAP after 30 min in phase 3. CAP prevents the induction of the autolysin (13).

Effect of mercuric chloride, 2-ME, and mercuric chloride plus 2-ME on the competent cell-associated protein. Since competence was fully restored in cultures treated with mercuric chloride and 2-ME during the first 10 min of phase 3 and not afterwards, and addition of CAP to cells treated similarly prevented competence from developing (Fig. 2), the possibility existed that mercuric chloride was, in some way, interfering with the synthesis of another competent cell-associated protein. Earlier studies showed that one did exist (14). Therefore, the effect of mercuric chloride, 2-ME, and mercuric chloride plus 2-ME on this protein was examined.

Figure 3 shows electrophoretograms of acidified phenol extracts of cells treated with CF and mercuric chloride in the presence or absence of 2-ME. These tracings are similar to those pub-

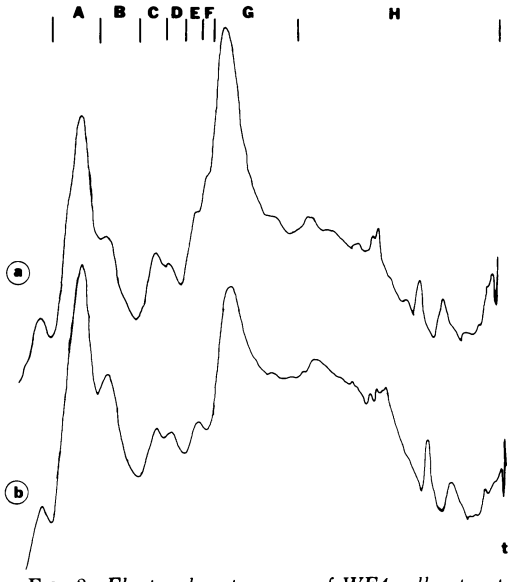

FIG. 3. *Electrophoretograms of WE4 cell extracts. (a) Cells treated with mercuric chloride (5 μM), 2-ME (20 mM), and CF (20%; vol/vol); (b) cells treated with mercuric chloride (5 μM) and CF (20%; vol/vol); treatments were for the duration of phase 3. The method used for preparing cell extracts and the method used for gel electrophoresis were already described (14). The letters designate quantitated fractions. t, Top of the separating gel. Stacking gels are not shown.*

lished earlier (14) for competent and noncompetent cells. Not all peaks are shown since some are eluted from the gels, at approximately 65 min, with the tracking dye. The competent cell associated protein is designated as peak F and is missing in cells treated with CF in the presence of mercuric chloride.

Peak F is also missing in extracts of the following: cells not treated with CF in the presence or absence of 2-ME, cells treated with CF and inhibitors of protein synthesis added at zero time or 10 min after the addition of CF in phase 3, cells treated with CF that had been digested with proteolytic enzymes, and cells treated with CF and NEM. Only those cells that are treated with active CF in phase 3 under conditions of uninhibited protein synthesis contain peak F, including nontransformable lysogenic WE4 cells (unpublished observation).

The above results show that mercuric chloride (or NEM) has a minimum of two affects on the competent state in WE4 cultures: (i) it prevents the function of the CF-induced autolysin, and (2) it prevents the manifestation of the CF-induced peak F protein. Because of this fortuitous observation, the relationship between them and competence could be directly studied.

Since peak F is not present in cells treated with CF and mercuric chloride at zero time in phase 3 and the autolysin is, it is doubtful that peak F is the autolysin.

Is peak F a product of autolytic activity? To answer this question, autolytic activity was permitted to occur in the absence of additional protein synthesis. To accomplish this, cells were treated in phase 3 with CF and mercuric chloride for exactly 30 min. At this time, cells received either 2-ME or 2-ME and an inhibitor of protein synthesis (e.g., CAP or rifampin) and were incubated at 37 C for an additional 20 min (total time in phase 3 was 50 min). The cells were then harvested by centrifugation, washed with saline, and extracted with acidified phenol (14). The extracts were subjected to PAGE. Another sample from each culture was tested for NaF-stimulated autolysis and for transformation (Table 4).

The data in Table 4 show that the amount of material present in each peak (except for peak F) is independent of the state of competence. Peak F varies from 0% for noncompetent cells (line 2) to 4.8% for fully competent cells (line 1). Line 3 shows that extracts derived from cells treated with CF and mercuric chloride in phase 3 do not contain peak F (Fig. 3). Line 4 shows that 20 min after the addition of 2-ME to mercuric chloride-inhibited cells the amount of material present in peak F increased from 0% (line 3) to 1.9%, and that 61% of the culture autolyzed. The data in line 5 show that, although 50% of this culture autolyzed, there was no increase in the amount of peak F material; rifampin prevented its synthesis. It is concluded that peak F does not arise from autolytic activity but from additional protein synthesis that is inhibited by mercuric chloride (or NEM). Line 6 shows data derived from a 2-ME-treated control culture. 2-ME does not influence the amount of material present in peak F even though these cells autolyzed to 71%. It should be pointed out that cells treated with CF and 2-ME during phase 3 do show a relative increase of about 40% in all acidified, phenol-extractable proteins over those cells that are only treated with CF. This 2-ME effect is not observed in cultures that are not treated with CF. CF, therefore, may stimulate all protein synthesis which is further enhanced by 2-ME or reducing agents in general. Line 7 shows that, during the first 30 min in phase 3, cells synthesize peak F to the extent of 2.8%. The kinetics of synthesis of peak F material is being studied. Note also in Table 4 that only cultures that received 2-ME autolyzed upon addition of NaF. In this experiment, cells were

TABLE 4. *Transformation, autolysis, and the distribution of cell proteins observed by polyacrylamide gel electrophoresis of extracts of WE4 cells*[a]

Treatment of WE4 cells in phase 3	Protein peak (% of total)								Transformation[b] (%)	Autolysis[c] (%)
	A	B	C	D	E	F	G	H		
1. CF and no $HgCl_2$ at zero time	15.5	11.9	6.0	5.0	5.8	4.8	26.7	24.4	0.37	16
2. No CF	21.7	14.0	5.3	4.2	4.4	0.0[d]	15.3	35.0	1×10^{-4}	10
3. CF and 5 μM $HgCl_2$ at zero time	12.1	8.8	4.8	3.8	4.2	0.0[d]	22.8	43.5	9.6×10^{-5}	0
4. CF and 5 μM $HgCl_2$ at zero time; 20 mM 2-ME added at 30 min	15.3	13.0	6.0	5.6	7.9	1.9	26.0	23.9	3.8×10^{-2}	61
5. CF and 5 μM $HgCl_2$ at zero time; 20 mM 2-ME and 1 μg of rifampin per ml added at 30 min	13.3	9.1	4.5	3.6	4.3	0.0[d]	28.5	36.9	1.9×10^{-3}	50
6. CF at zero time; 20 mM 2-ME added at 30 min	12.8	11.2	7.4	5.3	6.7	4.2	20.1	32.2	0.43	71
7. CF at zero time; 1 μg of rifampin per ml added at 30 min	12.6	6.7	4.9	2.8	4.2	2.8	23.8	42.0	0.23	7

[a] WE4 cells were grown for phases 1 and 2 in BHS broth (Difco) as described in Materials and Methods. The cells were treated in phase 3 as described. A sample from each culture was treated in phase 4 with a standard solution of DNA. Those cultures that did not receive rifampin during phase 3 were incubated with it during phase 4. Another sample was incubated with 10 mM NaF overnight at 37 C in order to observe autolysis. The remainder of the culture was harvested, washed, and extracted with phenol-acetic acid and water, and the extracts were subject to PAGE as described (14). Quantitation was done as described in Materials and Methods.

[b] See footnote *a* to Table 1.

[c] Percent autolysis is the decrease in optical density observed in cultures after overnight incubation.

[d] Below level of detection.

grown in phases 1 to 4 in BHS broth (BH from Difco).

Is peak F related to the competent state? A comparison of lines 4 and 5 in Table 4 shows that cells that contained peak F transformed to higher levels (20-fold higher) than those that did not, even though both cultures autolyzed about the same (50 to 60%). This difference in TF could be correlated with additional protein synthesis, which was further correlated with peak F. To show that an increased TF was dependent upon additional protein synthesis (and not just the reversal of the inhibition of autolytic activity; Fig. 2), the following was done.

Cells were incubated with CF and mercuric chloride for exactly 30 min in phase 3. At 30 min, 2-ME was added to reverse the inhibition. Rifampin (1 μg/ml) was then added at various times during phases 3 and 4 to prevent additional protein synthesis from occurring. After mercuric chloride inhibition was reversed by 2-ME, the TF increased over the next 50 min (Fig. 4). Therefore, to regain nearly full transforming activity, 50 min of uninhibited protein synthesis must occur (compare with Fig. 2).

This additional protein synthesis can be directly correlated with peak F (Table 5). The amount of material present in peak F 20 min after mercuric chloride inhibition was reversed by 2-ME was 1.9 to 3% (line 4, Table 4). After 50 min (80-min point in Fig. 4), the amount increased to 4.4%. Therefore, peak F is directly related to the competent state. Its function is yet unknown but may be related with irreversible binding of DNA to competent cells.

Is autolytic activity enough for cells to bind DNA or must additional protein synthesis also occur? To answer this question, cells were treated as described in the footnotes to Table 6 and assayed for both reversible and irreversible binding of tritiated DNA. Only the irreversible binding data are shown.

Line 1 shows that there was little irreversible binding of DNA by cells treated with CF and mercuric chloride during phases 3 and 4. When the inhibition was reversed by 2-ME, binding was a maximum (line 2). When the inhibition was reversed by 2-ME at 30 min in phase 3, the cells also took up DNA (line 3). (In this instance, protein synthesis was allowed to occur for 50 min after the inhibition of autolysis was removed.) Line 4 shows that reversal of the inhibition of autolytic activity in the absence of additional protein synthesis (see line 5, Table 4) was not enough to permit cells to bind large

TABLE 5. *Increase in the amount of peak F protein with time after reversing $HgCl_2$ inhibition of autolytic activity and protein synthesis with 2-ME*[a]

Treatment of WE4 cells in phase 3	Protein peak (% of total)							
	A	B	C	D	E	F	G	H
1. CF and 5 μM $HgCl_2$ at zero time; 20 mM 2-ME added at 30 min for 20 min	12.8	10.4	5.1	5.8	5.8	3.0	21.8	35.4
2. CF and 5 μM $HgCl_2$ at zero time; 20 mM 2-ME added at 30 min for 50 min	12.4	12.0	6.8	5.8	7.1	4.4	22.5	29.1

[a] WE4 cells were treated as described in the footnotes of Table 4 and as above. Quantitative data were obtained by the procedures described in Materials and Methods.

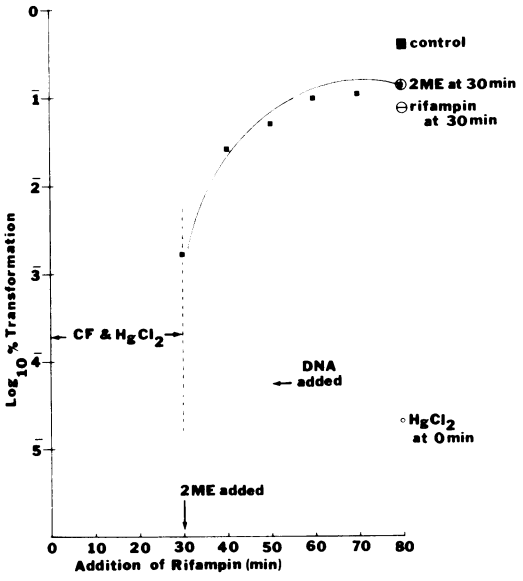

FIG. 4. *Kinetics of reversal of mercuric chloride inhibition of competence development by reversing inhibition of autolytic activity and protein synthesis with 2-ME. WE4 cells were grown in phases 1 and 2 in BHS broth (Difco). The cells were treated with CF (20%; vol/vol) and mercuric chloride (5 μM) for exactly 30 min at 37 C in phase 3. At 30 min, 20 mM 2-ME was added. Also starting at the 30th min, the cells were treated further with rifampin (1 μg/ml). At the end of phase 3 (50 min), all cells were added to a standard DNA solution. Incubation and the addition of rifampin were continued for an additional 30 min (to the end of phase 4). Phases 5 and 6 were carried out as described. The points labeled "control," "2-ME at 30 min," and "rifampin at 30 min," are the transformation frequencies obtained by cultures treated in the described manner (in phases 3 and 4) with CF in the absence of mercuric chloride. For the point labeled "$HgCl_2$ at 0 min," cells were treated in phases 3 and 4 with CF and mercuric chloride at the above concentrations.*

amounts of DNA irreversibly. Additional protein synthesis must first occur (lines 5, 6, and 7). Lines 8, 9, and 10 are various control cultures.

Is peak F associated with reversible binding of DNA? The answer to this question at this time is no, since lysogenic cells that have been put through a competence regimen do not bind DNA reversibly (8) even though they contain a substantial amount of peak F protein (unpublished observation) and are capable of autolyzing maximally (8). These observations define the reversible DNA binding site which is also thought to be protein (5).

DISCUSSION

It is a generally accepted fact that the development of competence requires protein synthesis (18). Two recent discoveries, made in cultures of the group H streptococci, that relate directly with de novo protein synthesis and the competent state were: (i) the demonstration of a new protein (designated here as peak F) after polyacrylamide gel electrophoresis of acidified phenol extracts of competent or potentially competent cells (14), and (ii) the demonstration of an autolysin that is also present only in competent or potentially competent cells (13). Potentially competent cells are those that are treated with CF but do not go on to transform because they cannot bind DNA reversibly (e.g., some lysogenized cultures; 8).

Earlier kinetic studies (9) showed that a culture's potential to autolyze occurred simultaneously with its potential to transform (develop competence). This work was initiated, therefore, to test directly whether the competent state was dependent upon an autolytic event.

Because maximal autolytic activity required a reducing agent, the need for sulfhydryl groups was inferred. Therefore, chemicals that combine with sulfhydryl groups (mercuric chloride, NEM) were added to cells that were treated with CF, and their affect on the development of competence (transformation) and autolysis was observed. When added at an early time period, these chemicals inhibited the development of competence (transformation; Tables 1 and 2).

They also inhibited autolysis (Fig. 1; data for NEM not shown). Moreover, inhibition of both events could be reversed by 2-ME (Table 3, Fig. 1, 2, and 4). Reversal of just the inhibition of autolytic activity by 2-ME caused a 66-fold increase in the level of transformation above the mercuric chloride-treated control (Fig. 2; see also the 30-min point in Fig. 4). This result supports the hypothesis that competence development requires a local, limited autolytic event. However, the results also showed that full recovery from mercuric chloride inhibition required additional protein synthesis (Fig. 2, 4; Table 4). This was manifested as the protein in peak F and the protein in the reversible DNA binding site(s) (5).

The differential inhibition of CF-induced proteins exhibited by both NEM and mercuric chloride was unexpected and was used to answer other specific, competence-related questions. Since the autolysin was synthesized almost maximally in the presence of either 50 μM NEM or 5 μM mercuric chloride and the other two proteins were not, it was shown that peak F was not the autolysin as originally surmised (13). Moreover, peak F was shown not to be the result of autolytic activity (Table 4). Its concentration increased with continued protein synthesis and was proportional to the frequency of transformation (competence, Table 5). The data also showed that autolytic activity alone was not enough to allow cells to react with DNA (Table 6).

The above differential effect on induced protein synthesis shown by both mercuric chloride and NEM can be explained if neither reagent readily penetrates into the cell's cytoplasm. Since both chemicals behave in an identical fashion, they are probably acting on the same sites: the sulfhydryl groups in specific ribosomal proteins (15) and the sulfhydryl groups in the autolysin molecule. It is assumed that the protein in peak F and in the DNA binding site(s) are synthesized on membrane-bound ribosomes (readily accessible to mercuric chloride or NEM), whereas the autolysin is synthesized on cytoplasmic ribosomes (not readily accessible to mercuric chloride or NEM). Membrane-bound ribosomes and cytoplasmic ribosomes have been separated and characterized (K. Nugent and T. S. Theodore, personal communication; 2, 16). This inhibition, therefore, is different from CAP inhibition, for example, which affects all protein biosynthesis since it is readily permeable to the cells.

The differential inhibition of de novo protein synthesis can also be explained in terms other than permeability. Alkylated sulfhydryl groups on ribosomes or initiation factors may alter their specificity for recognizing certain messenger ribonucleic acids while retaining selective specificity for others (17). Therefore, two separate messengers are indicated.

The fact that competent cells or potentially competent cells lyse in certain broth media (BBL brain heart infusion) but not in other broth media (Difco brain heart infusion), unless supplemented with a reducing agent, in the presence of an inhibitor of energy metabolism (fluoride or arsenate), probably means that autolysis and cell wall resynthesis are in a "conditional steady state" of limited duration.

TABLE 6. *DNA binding by WE4 cells treated in various ways in phase 3*[a]

Treatment of WE4 cells	Counts per 10 min
1. CF and 5 μM $HgCl_2$ at zero time	514
2. CF and 5 μM $HgCl_2$ at zero time; 20 mM 2-ME added at zero time	2,889
3. CF and 5 μM $HgCl_2$ at zero time; 20 mM 2-ME added at 30 min	1,204
4. CF and 5 μM $HgCl_2$ at zero time; 20 mM 2-ME and rifampin (1 μg/ml) added at 30 min	406
5. CF and 5 μM $HgCl_2$ at zero time; 20 mM 2-ME added at 30 min; rifampin (1 μg/ml) added at 50 min	630
6. CF and 5 μM $HgCl_2$ at zero time; 20 mM 2-ME added at 30 min; rifampin (1 μg/ml) added at 70 min[b]	943
7. CF and 5 μM $HgCl_2$ at zero time; 20 mM 2-ME added at 30 min; rifampin (1 μg/ml) added at 80 min[b]	846
8. CF; 20 mM 2-ME added at 30 min	2,408
9. No CF; 20 mM 2-ME added at 30 min	270
10. CF; DNA first digested with pancreatic deoxyribonuclease (10 μg/ml)	193

[a] WE4 cells were grown in phases 1 and 2 in BHS broth (Difco) as described in Materials and Methods. The cells were treated in phase 3 as described above, before the addition of [H^3]DNA in phase 4. Radioactivity was measured as described in Materials and Methods.

[b] Rifampin added after [H^3]DNA addition. Background counts (about 120 to 150 per 10 min) were not subtracted from the above data.

The condition and the duration depends on the availability of the cell wall substrate and the availability of ATP for energy or biosynthesis. Competent or potentially competent cells can also be made to autolyze if they are incubated in BHS (BBL) broth at nonpermissive growth temperatures: room temperature or below. It is concluded that the CF-induced autolysin is degradative and that its sole function is to transiently expose membrane-associated DNA binding sites (peak F protein?).

One group of investigators (4) recently reported that they could not demonstrate peak F after PAGE of acidified phenol extracts of competent cells. They used stacking gels consisting of a different chemical composition than the one presented here and elsewhere (14). The physical consistency and the chemical composition of the stacking gel appear to be important for peak F to be manifest (3). Stacking gels prepared from old stock solutions, or stock solutions kept in glass bottles, or both, do not permit resolution of peak F (unpublished observations). New solutions stored in plastic bottles present no problem. Gels run without stacking gels do not resolve the protein in question. Because of these differences, the gel patterns described by Fuchs et al. are also very much different from the ones presented here (Fig. 3) and elsewhere (14).

The results presented here also differ from those obtained with *Bacillus subtilis*. In *B. subtilis* cultures, mercuric chloride interferes with transformation by preventing the attachment of DNA to the competent cell (Groves and Young, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 58, 1973; F. Young, personal communication).

In general, the results presented in this report bring together a number of independent observations and show their interrelationships with the overall picture of competence in group H streptococci. A loss of any one function can render the cell noncompetent. How the cell treats DNA after it is taken up and whether these events require other new proteins remain to be answered.

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