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Cellular Metabolism in Genetic Transformation of Pneumococci: Requirement for Protein Synthesis During Induction of Competence

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Metabolic inhibitors have differential effects on various phases of genetic transformation in pneumococci. Evidence is presented suggesting that, in addition to the competence factor, another specific protein or class of proteins is essential for the development of cellular "competence." The precise role of this protein(s) in genetic transformation is not known, but it seems essential for some function subsequent to the interaction of competence factor and cells.

During genetic transformation deoxyribonucleic acid (DNA) molecules of several million molecular weight are transported through the surface membranes of pneumococci. To a certain extent, this process is analogous to the penetration of cell surfaces by genetic material during virus infection or during sexual conjugation of enteric bacteria. However, in contrast to these latter phenomena, in transformation the donor is a bare DNA molecule, and thus the whole apparatus for the cellular uptake of DNA molecules must reside in the recipient bacterium. In addition, these bacteria must be in a "competent" physiological state.

Investigations into the biochemical nature of this important physiological state were facilitated by the recent isolation of macromolecular "competence factors" which can induce the competent state in the homologous bacteria (1, 5, 6, 13, 21).

The pneumococcal competence factor (CF) appears to be a basic protein (or protein-containing substance) with a molecular weight of approximately 10,000 (22). The interaction of this substance with pneumococci results in the rapid development of the competent state in over 90% of the cell population (10). We tried to learn more about the interaction of this factor with the bacteria as well as about the biochemical nature of the competent condition by testing the effect of selective metabolic inhibitors on pneumococci during and after their interaction with the CF.

MATERIALS AND METHODS

Design of experiments. Variations of a single basic procedure were used in all experiments. The general structure and rationale of the experiments were as follows.

Phase 1. Cultures of pneumococci were grown in a chemically defined (CD) medium at *p*H 6.8. (A. Tomasz, Bacteriol. Proc., p. 52, 1967). At this *p*H, the cultures grow with normal generation times and reach normal stationary cell concentration, but they fail to produce endogenous CF and remain completely incompetent (20). This *p*H is also far below optimal for the action of CF.

Phase 2. When the culture reached the desired cell concentration in low *p*H medium, the *p*H was shifted to 8 (optimal level for CF) by the addition of alkali, and purified CF was added (less than 0.1 μg of protein per 10 ml of medium) to induce the competent state. After such a *p*H shift, bacteria can be converted to competence without any detectable time lag by the addition of suitable concentrations of CF. Without the addition of CF, spontaneous expression of competence requires an induction period, the length of which is an inverse function of the cell concentration, presumably because the bacteria must first produce endogenous CF (20).

Phase 3. The bacteria were transferred to a fresh medium, by one of several alternative methods, such as 10× dilution, membrane filtration (Millipore Corp., Bedford, Mass.), or centrifugation. The bacteria were incubated for various periods to test the effect of the particular medium on the stability of competence.

Phase 4. The bacteria were diluted 10× into fresh medium to which transforming DNA was added (0.1 to 0.5 g/ml) plus a low concentration (0.1 μg/ml) of subtilisin (to prevent further action of residual CF). In some experiments, the action of the CF was terminated by shifting the *p*H of the medium back to 6.8. [These conditions do not seem to interfere with transformation of the already competent bacteria during relatively short (10 to 20 min) exposure to

DNA.] After 10 to 20 min of incubation with transforming DNA, pancreatic deoxyribonuclease was added (1 to 3 μg/ml). Transformants were scored by conventional techniques (9).

Treatments of bacteria with drugs or with inhibitory conditions (e.g., deprivation of a required nutrient) were always restricted to a single phase of the experiment, i.e., the inhibitors were removed or the cells were transferred to fresh, complete growth medium before the start of the next phase of the experiment, or both.

In all the isotope incorporation experiments, the samples were extracted with cold 10% trichloroacetic acid prior to counting. Thus, incorporation data refer to isotope incorporated into cold acid-insoluble material (protein or nucleic acid.

Measurement of the induction of competent state by purified CF. For the quantitative evaluation of drug inhibition experiments, it was essential to understand better the kinetics of interaction between CF and the bacteria. Fig. 1 shows that, upon the addition of purified CF, conversion to the competent state commences without an appreciable time lag. The dependence of the overall rate of competence induction on both the concentration of the incompetent cells and the concentration of the CF is documented in Fig. 2 and 3. In the first of these two experiments, a series of concentrations of incompetent bacteria (ranging from 10^4 to 10^8 cells per ml) was treated with CF. After 5 min, the cell suspensions were diluted with appropriate volumes of fresh medium so as to yield identical cell concentrations. Subtilisin (0.1 μg/ml) and saturating concentrations of transforming DNA were added. In the second experiment, the concentration of the CF was varied. Both curves (Fig. 2 and 3) show a range of saturation in which the initial rates of conversion to competence reach a maximal value. It should be emphasized that the values plotted represent initial rates: the concentration of at least one of the "reactants," the incompetent cells, may be considered only insignificantly changed, since during the time span of the experiments the conversion of the cells to competence was never more than 15% of the maximal. In Fig. 2 and 3, the initial rate of conversion to competence is plotted as a function of cell concentration (Fig. 2, slope = 1) or as a function of the concentration of CF (Fig. 3, slope = 3 to 3.5). Saturating concentrations of CF were used in all experiments.

Titration of CF. Titration of CF in cultures was based on the finding that the initial rate of conversion of pneumococci to competence is a function of the concentration of CF. The titration of CF was performed in the following way. A 0.1-ml amount of the suspension to be assayed (containing live pneumococci of mutant A as well as their growth medium) was added to a 0.9-ml suspension of incompetent pneumococci (mutant B) "marked" with some genetic property such that, on selective media, they could be scored separately from the cells of mutant A. After a short incubation, portions of this mixed culture were diluted into fresh growth medium containing subtilisin and transforming DNA, and transformants of mutant B were scored on selective medium.

In all experiments, derivatives of the rough strain

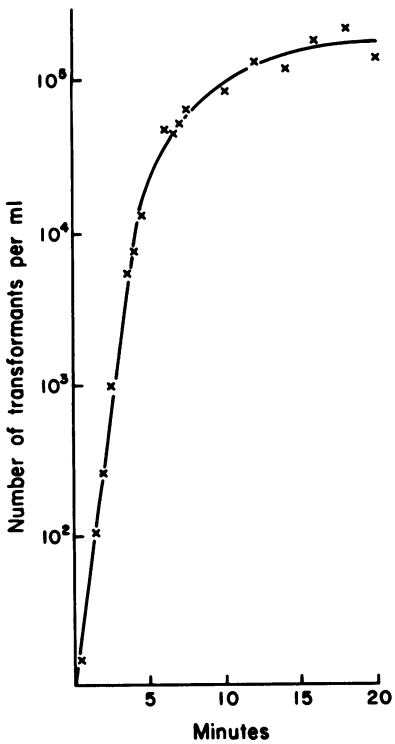

FIG. 1. *Induction of competence by purified competence factor.*

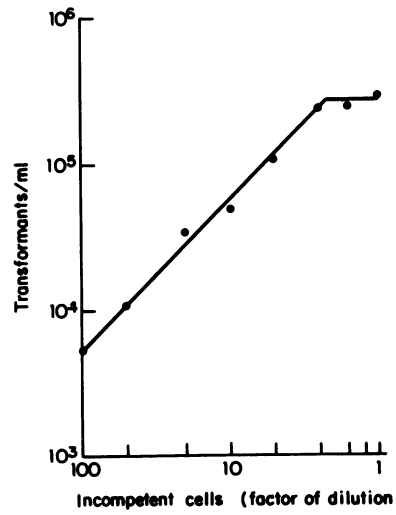

FIG. 2. *Induction of competence: the initial rate of induction as a function of the concentration of incompetent cells.*

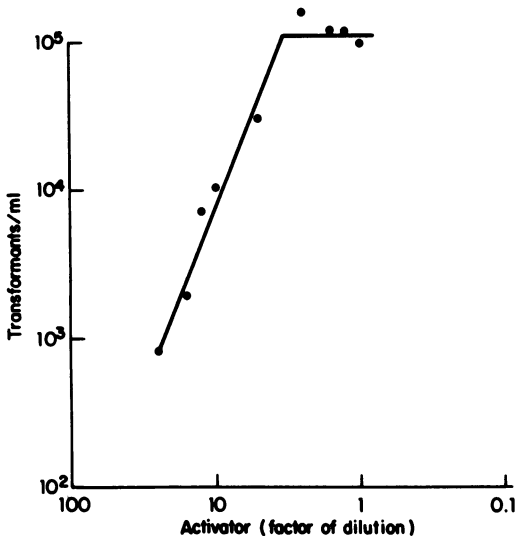

FIG. 3. *Induction of competence: the initial rate of induction as a function of competence factor concentration.*

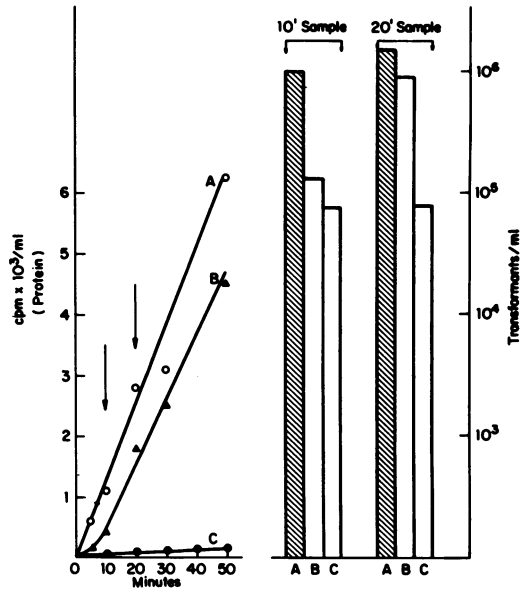

FIG. 4. *Effect of valine starvation on the induction of competence. Left side shows the rate of protein synthesis (incorporation of ^{14}C-phenylalanine) in a control culture (tube A), a valine-starved culture in the absence of valine (tube C), and a valine-starved culture after readdition of valine (tube B). CF was added to the cultures at zero minute, and the number of transformable bacteria was determined at the times indicated by the arrows; the histograms on the right side of figure show the results.*

of *Diplococcus pneumoniae* R6 (originating from strain R36A) were used. R6T-9 is a thymidine-dependent derivative of R6. It was constructed by transforming a competent culture of R6 with DNA isolated from a thymidine-dependent mutant strain of pneumococcus and selecting for thymidine-dependent transformants. The mutant strain was isolated and kindly provided by H. Friedman in A. Ravin's laboratory.

Media and procedures used for the growth and counting of bacteria were described in earlier publications (20). Measurements of radioactivity were done by liquid scintillation counting (Unilux I and Mark I spectrometers; Nuclear-Chicago Corp., Des Plaines, Ill.).

All chemicals used were reagent grade commercially available products. Tryptazan was a gift from E. W Balis of the Sloan-Kettering Institute for Cancer Research, N.Y. Rifamycin was kindly provided for by H. Fromageot of this University.

RESULTS

Effect of amino acid deprival on the induction of competence. Inhibition of the induction process by amino acid deprivation has already been briefly described (22). Figure 4 depicts such an experiment in detail. A culture of bacteria grown in the low *p*H medium (phase 1) was transferred to the same medium lacking the required amino acid (valine), and the culture was incubated for 40 min to deplete intracellular pools of this amino acid. A culture with a full complement of valine was also incubated as a control (tube A). At the end of the depletion period, the control culture was diluted back to the cell concentration of the valine-limited culture. The *p*H was shifted to 8, CF was added [in the ratio of 1:10 (v/v) to cell suspension] together with radioactive phenylalanine (0.01 to 0.1 μc per ml, 5 to 10 μg per ml). The valine-limited culture was immediately distributed into two tubes: a normal concentration of valine (340 μg/ml) was added to tube B, whereas the tube C remained unsupplemented. The three tubes were incubated at 30 C, and samples were withdrawn periodically to determine the amount of isotope incorporated into protein and to score the number of transformants. For the latter assay, 0.1-ml portions of the cultures were diluted into 0.9 ml of fresh CD medium, complete with valine and containing subtilisin and transforming DNA. Figure 4 shows that valine starvation during the interaction of the bacteria with the CF caused an over 90% inhibition of the induction process. The data also show that the inhibitory effects could be quickly and nearly completely reversed by the readdition of valine.

Effect of chloramphenicol (CAP), puromycin, and tryptazan on the induction of competence. This effect is demonstrated in Fig. 5 and 6. In the CAP experiment, the drug (25 μg/ml) was added

to portions of a culture already in the *p*H 8 medium (phase 2), but either 10 min before (in experimental tube C) or 5 min after (in tube B) the addition of CF. A control tube (A) received no CAP. The incorporation of radioactive phenylalanine was used to monitor the rate of protein synthesis. Fig. 5 demonstrates three essential points. (i) Inhibition of protein synthesis by CAP during the interaction of cells and CF completely inhibits the acquisition of competence. (ii) Apparently, a continuous protein synthesis is required, since addition of CAP 5 min after the initiation of the induction process (tube B) results in an immediate cessation of further conversion to competence. In fact, the competence already achieved *before* the addition of the drug is also rapidly lost under these conditions, suggesting that the maintenance of the competent state—and not only its acquisition—also requires protein synthesis. (iii) When CAP was removed from a portion of culture B by centrifugation (tube BB, Fig. 5) and the cells were resuspended in fresh medium containing the isotope and CF, the bacteria rapidly recovered their ability to react with the CF, parallel to the resumption of cellular protein synthesis.

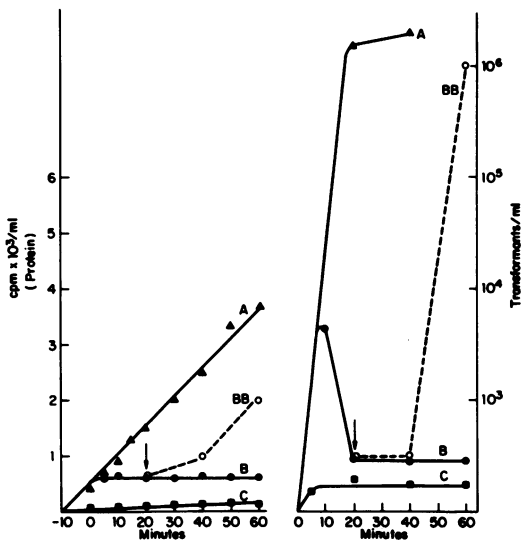

FIG. 5. *Effect of chloramphenicol on the induction of competence. Left side shows rate of protein synthesis (incorporation of ^{14}C-phenylalanine) in a control culture (tube A) and in cultures to which chloramphenicol was added 10 min before (tube C) or 5 min after addition of CF (tube B). From a portion of culture B, the drug was removed at the time indicated by the arrow (tube BB); after resuspension in fresh medium, cells in tube BB received fresh isotope and CF. Right side of figure shows cellular competence in tubes A through C. Before adding the transforming DNA, each sample was transferred to drug-free medium.*

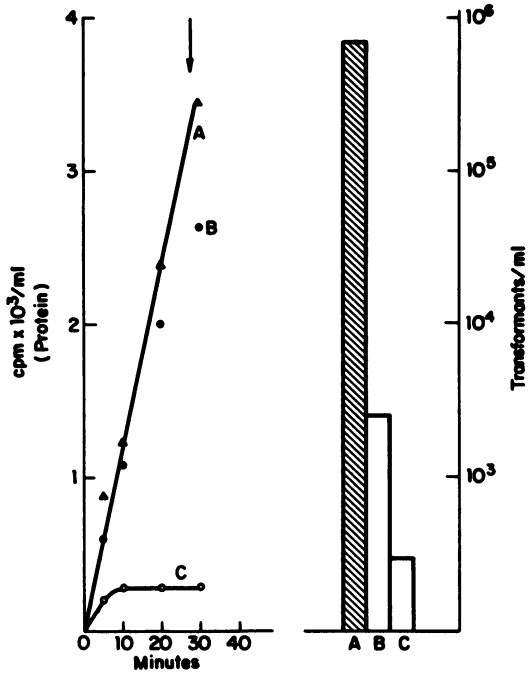

FIG. 6. *Effect of puromycin and tryptazan on the induction of competence. Left side of figure shows rates of protein synthesis in a control culture (tube A) and in cultures to which tryptazan (tube B) or puromycin (tube C) were added at zero minute, together with CF. At the time indicated by the arrow, samples were removed to drug-free medium, and the number of transformable cells was determined (right side).*

Part of Fig. 6 shows an identical experiment with puromycin (100 μg/ml). Again the inhibition of protein synthesis is paralleled by a complete inhibition of the process of competence induction.

The second part of Fig. 6 summarizes a similar experiment in which the tryptophan analogue, tryptazan, was used. This amino acid analogue does not inhibit protein synthesis (4). Rather, it was shown to incorporate into protein, replacing tryptophane (15). It has been reported that incorporation of this amino acid into the coat protein of phage T2 results in an abnormal phage protein with a lowered capacity to adsorb to sensitive cells (16). Figure 6 shows that, as expected, exposure of pneumococci to tryptazan during the induction process did not inhibit formation of protein. On the other hand, the acquisition of competent state was severely inhibited. The inhibition could be prevented by the simultaneous addition of equimolar concentrations of tryptophan.

Effect of rifamycin on the induction of competence. This effect is demonstrated in Fig. 7 and 8. Both the design of the experiments as well as the results are completely analogous to those illustrated in Fig. 5 and 6. Rifamycin (0.1 μg/ml; 12) was added to portions of a culture at the beginning of phase 2 of the experiments; tube C received the drug together with the CF, in tube B the drug was added 5 min after the CF, and tube A was the control. Figure 7 shows that (i) inhibition of ribonucleic acid (RNA) synthesis inhibited induction of competence, and (ii), just as in the case of inhibitors of protein synthesis, continuous RNA synthesis was required both for the acquisition and the maintenance of cellular competence. Figure 8 demonstrates the rapid reversibility of inhibition. A culture at the beginning of phase 2 (prior to addition of CF) was halved; rifamycin was added to one tube, whereas the other served as a control. After 10 min of incubation, the bacteria were filtered, washed (to remove the drug), and resuspended in fresh phase 2 medium with CF, and radioactive uracil was added for monitoring RNA synthesis. The quick recovery of the cells' reactivity to CF parallels the resumption of RNA synthesis.

Induction of competence in cultures with slow growth rates. In the R6 strain of pneumococcus, lowering of the valine concentration of the growth medium results in a lower net growth rate. A pyrimidine-requiring mutant of pneumococcus responds similarly to the lowering of uridine concentration in the medium. In both cases, the

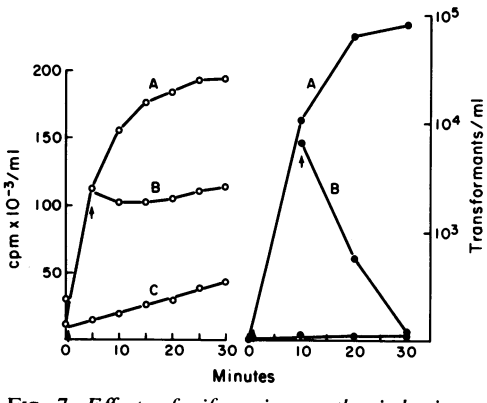

FIG. 7. *Effect of rifamycin on the induction of competence. Left side shows RNA synthesis (incorporation of ^{14}C-uracil) in a control culture (tube A) and in cultures to which rifamycin was added at times indicated by the arrows: at zero minute (tube C) and at 5 min (tube B). All cultures received CF at zero minute. The number of transformable cells was determined after various times of incubation (right side).*

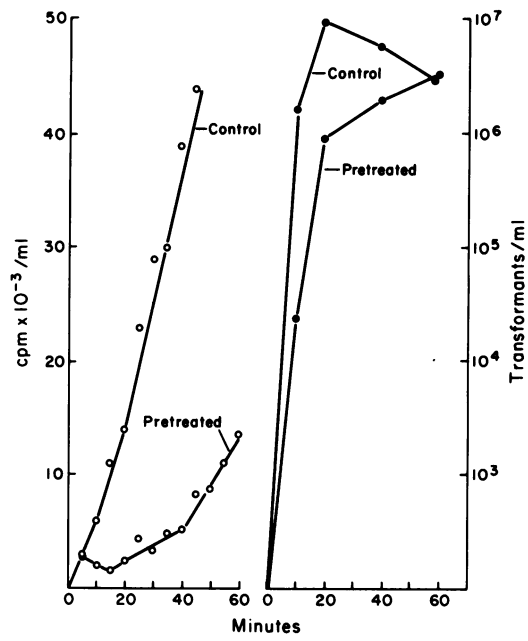

FIG. 8. *Recovery of RNA synthesis and cellular reactivity to CF after removal of rifamycin. Left side shows RNA synthesis in a control culture and in a culture which was pretreated with rifamycin for 10 min. At zero minute of the experiment, the drug-treated culture was transferred to drug-free medium. Isotope (^{14}C-uracil) and CF were added to the cultures at zero minute, and the number of transformable bacteria was determined at intervals (right side).*

bacteria seem to have a defect in the concentrative uptake of the nutrients from the medium (20). Figures 9 and 10 demonstrate that one can exploit this property of the bacteria to produce cultures with different rates of net protein synthesis. The cultures at the end of the usual phase 1 were centrifuged, washed, and resuspended in valine-free or uridine-free medium still at *p*H 6.8 and incubated at 37 C (to deplete intracellular pools of these nutrients). The *p*H was then shifted to 8, and portions of the cultures were supplemented with different concentrations of valine or of uridine, respectively. Purified CF and radioactive phenylalanine were added, and the rate of conversion of cells to competence as well as the rate of protein synthesis was followed. Figures 9 and 10 show a striking parallel between the rates of isotope incorporation to protein and the rates of conversion to competence.

Effect of inhibition of DNA synthesis on the induction of competence. This effect was tested by using the inhibitor flourodeoxyuridine (FUdR) or by thymidine starvation of a thymidine-requiring strain of pneumococcus. The "reactivity" of bac-

teria with CF was tested after various periods of thymidine starvation or FUdR treatment in the phase 2 medium. Thymidine was added back to the cells and FUdR was removed at the time of addition of transforming DNA. Figures 11 and 12 show that severe inhibition of DNA synthesis (80 to 95%) had little if any inhibitory effect on the reactivity of cells to CF. In fact slight stimulating effects were occasionally observed.

Separation of the protein synthesis-requiring step from the interaction of cell and CF. One of the main conclusions that can be drawn from the experiments described so far is that the addition of CF to incompetent cells only yields competent bacteria when the synthesis of some protein(s) is allowed to take place in the cells *after* the addition of CF. We tried to resolve this process into separate steps by modifying the experimental design in the following manner. First, incompetent cells were treated with CF in the absence of protein synthesis, and then, after the removal of excess cell free CF, protein synthesis was allowed to resume in the presence of subtilisin or at low *p*H, i.e., under conditions in which cell-to-cell propagation of the competent state would be excluded. The appearance of competent cells in this subtilisin-containing (or low *p*H) medium was monitored. The detailed design of the experiment was as follows. The protein synthesis of a culture at the start of phase 2 (but prior to the addition of CF) was inhibited either by the addition of CAP or by transfer of the cells to leucine-free medium. Ten minutes later the culture was halved: to one tube a saturating concentration of CF was added (tube B), the other tube served as a control (tube A). After 20 min of incubation at 37 C, both cultures were centrifuged; the pellets were rinsed with growth medium, and each was then resuspended in three different kinds of media: *p*H 8 growth medium containing subtilisin (A_1 and B_1), *p*H 8 growth medium without subtilisin (A_2 and B_2), and low *p*H (6.8) medium (A_3 and B_3). All tubes were incubated at 30 C, and 0.1-ml portions were removed at intervals into tubes containing 0.8 ml of *p*H 8 medium, subtilisin, and transforming DNA to test the numbers of competent cells. Transformations were terminated after 10 min. At the same time intervals, additional separate samples were removed from tube B_2 to titrate the CF content of this culture. Figures 13 and 14 clearly demonstrate that restoration of protein synthesis allowed the development of competence in all of the B tubes but in none of the A tubes. The important part of this observation is the development of nearly normal levels (and rates) of competence in tubes B_1 and B_3, i.e., under the conditions in which induction of competence by CF *accidentally* carried over from the

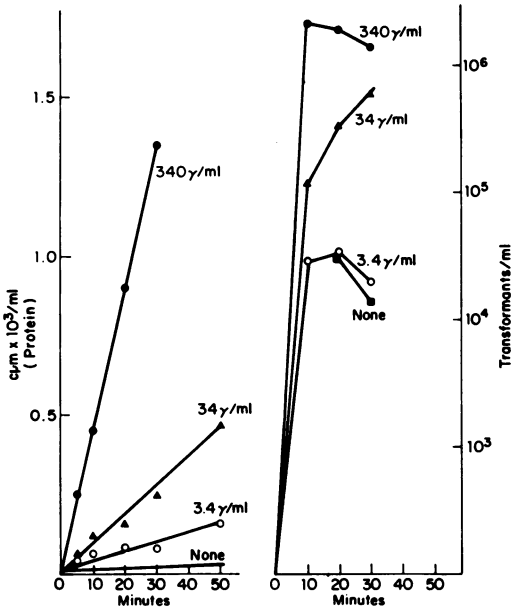

FIG. 9. *Induction of competence in cultures with different growth rates; valine-limited growth. Incompetent pneumococci were inoculated into a series of tubes containing phase II media, each with a different concentration of valine. Left side shows the rate of protein synthesis (incorporation of ^{14}C-phenylalanine). The right side shows induction of competence in the same cultures by CF added at zero minute.*

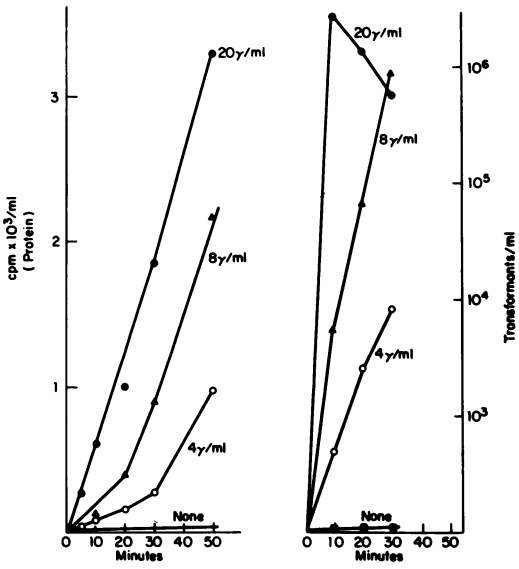

FIG. 10. *Induction of competence in cultures with different growth rates; uridine-limited growth. The design of the experiment was the same as that in Fig. 9, except that the growth media differed from one another in uridine concentration.*

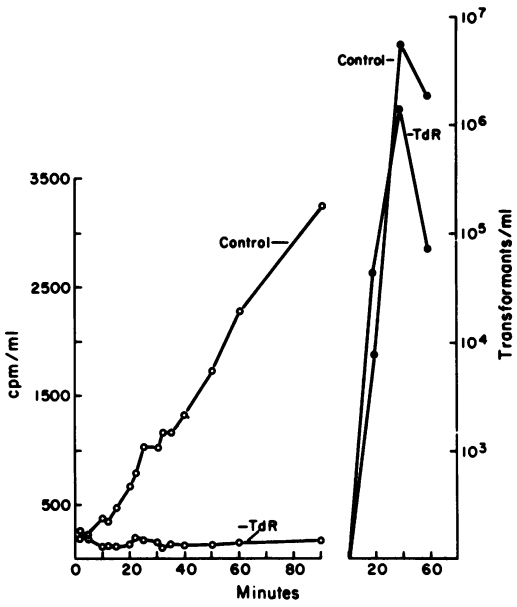

FIG. 11. *Inhibition of DNA synthesis by thymidine starvation; effect on induction of competence. Left side shows DNA synthesis (incorporation of ^{14}C-deoxyuridine) in a thymidine-requiring strain of pneumococcus with and without thymidine in the growth medium. The right side shows induction of competence in the same cultures by CF added at zero minute.*

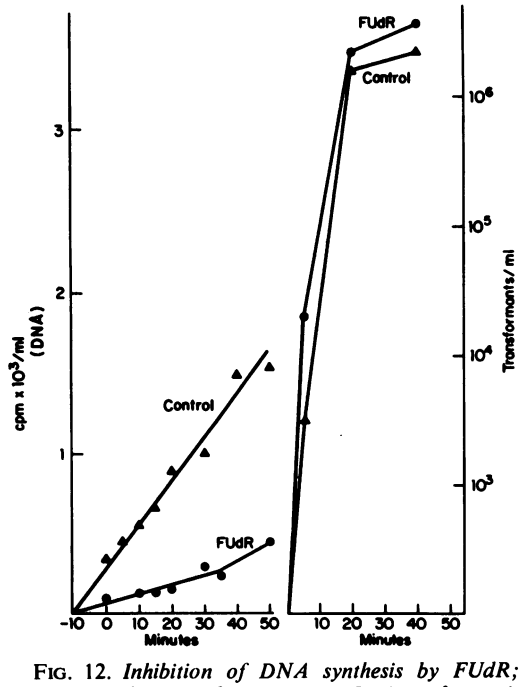

FIG. 12. *Inhibition of DNA synthesis by FUdR; effect on induction of competence. Design of experiment same as in Fig. 11, except that DNA synthesis was inhibited by the addition of 20 μg of FUdR per ml.*

first incubation medium is excluded. Growth conditions in tube A_2 allow spontaneous development of competence (*see* Fig. 14) with a rate which depends on the concentration of cells and on the cells' recovery from the inhibition of protein synthesis.

Thus, it appears that bacteria with inhibited protein synthesis could nevertheless bind CF, or interact with CF, in some manner such that afterwards, upon restoration of protein synthesis, the competent state could develop. It is important to emphasize that the appearance of competence under these conditions required incubation of the previously inhibited bacteria; i.e., immediately

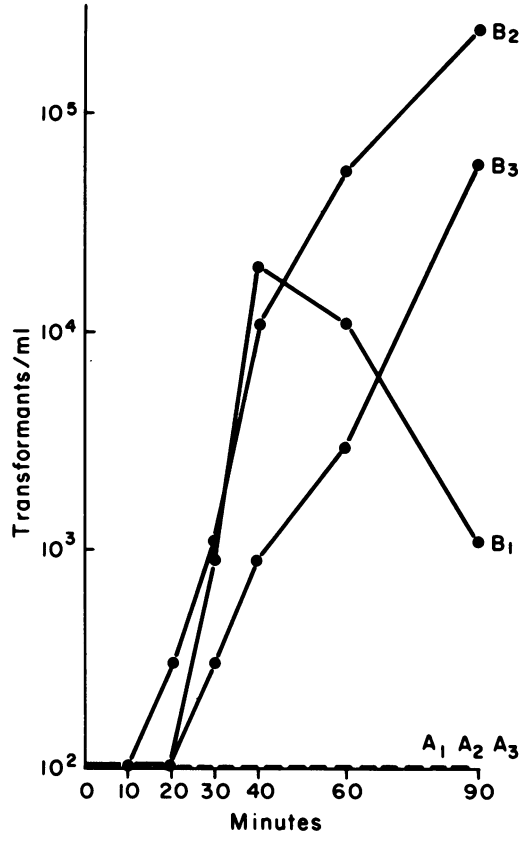

FIG. 13. *Separation of a protein synthesis requiring step from the interaction of cell and CF (A). A culture of pneumococci in phase II medium received 25 μg of chloramphenicol per ml; after 10 min of incubation, half of this culture (B) also received CF; 20 min later both cultures A and B were centrifuged and washed to remove CF and chloramphenicol. The cells were resuspended in three kinds of media: in phase II medium with subtilisin (A_1 and B_1); in phase II medium without subtilisin (A_2 and B_2); and in phase I medium (pH 6.8, A_3 and B_3). The graphs show the development of competent state in the six cultures during incubation at 37 C.*

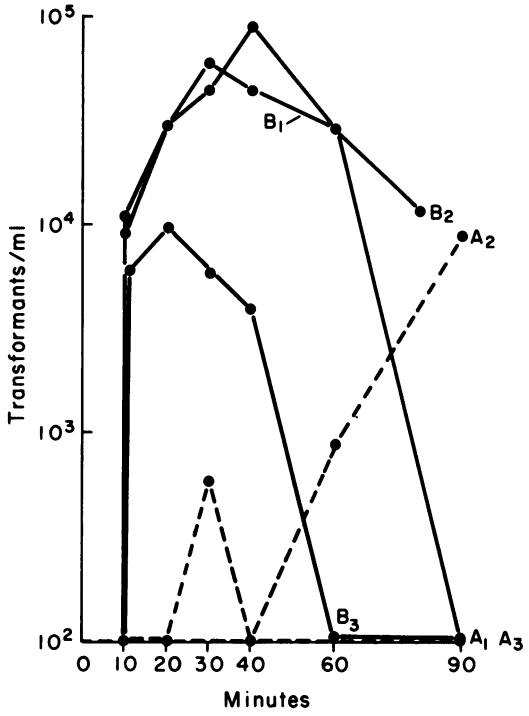

FIG. 14. *Separation of the protein synthesis-requiring step from the interaction of cell and CF (B). Design of experiment same as in Fig. 13, except that leucine starvation was used instead of chloramphenicol to inhibit protein synthesis.*

after the removal of CAP, the cells were still incompetent.

Titration of CF in culture B_2 revealed the presence of measurable quantities of cell bound CF (Fig. 15). Most importantly, the titer of CF in such cultures did not change or actually even dropped at the very times when the number of competent cells in the culture increased greatly. It should be recalled that under the "normal" conditions of assay, namely, during the induction of competence in growth media which allow simultaneous protein synthesis, the appearance of competent cells is also invariably accompanied by a great increase in the titer of CF (20). The finding of unchanging or even decreasing CF titer during the very substantial increase in the number of competent cells is strikingly different from the usual behavior of this system. It suggests that the appearance of competent state in the B cultures occurs via the completion of a two-step process, the first step of which is binding (or some interaction, or both) of CF to bacteria, and a second step, the synthesis of some critical protein molecule *other* than the CF itself.

Maintenance of the competent state. The inherent lability of the competent state in individual pneumococci has been known for considerable time (19). It was estimated that, in exponentially growing cultures at 37 C in broth medium, average pneumococci remain competent only for 20 to 30% of their generation times (8, 19). The cause of this lability is not known. We performed some experiments concerning the metabolic requirements for the maintenance of the competent state. A culture was made competent with CF and, at the end of phase 2, the cells were resuspended in four different kinds of media, all at *p*H 8: tube 1, in complete growth medium; tube 2, in medium lacking the main sources of energy (glucose and sucrose); tube 3, in medium lacking leucine; and tube 4 in medium lacking leucine, glucose, and sucrose. The bacteria were incubated at 30 C, and the number of competent cells was determined at time intervals. In tube 3, the titer of CF was also followed. Figure 16 shows that (i) omission of leucine from the medium greatly accelerated the spontaneous decline of competence (tube 3), (ii) omission of an energy source had no effect on the stability of competence (tube 2), and (iii) omission of an energy source has actually provided protection against the decay caused by the minus leucine condition. The interpretation of these quite complex findings in biochemical terms is not possible at the present time. Several formal conclusions, however, can be made. First, the "maintenance" of cellular competence apparently requires continued protein synthesis. Second, the "decay" of competence seems to occur by an active process requiring

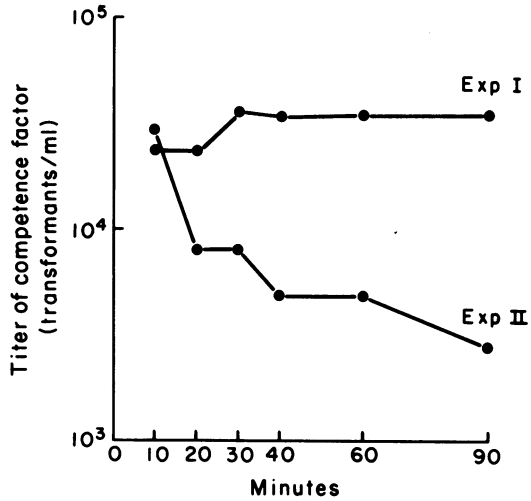

FIG. 15. *Titration of CF concentration. The concentration of CF in culture B_2 of the experiment in Figure 13 was determined at various times during development of competence. See the legend of Fig. 13 for more details.*

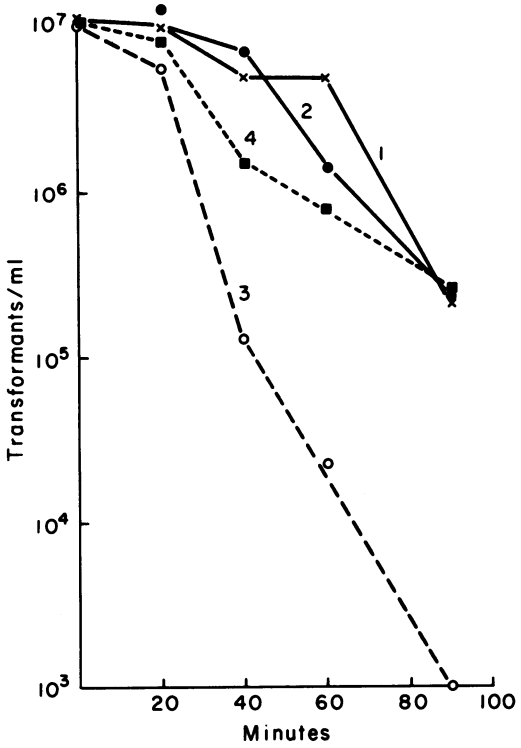

FIG. 16. *Effect of metabolic inhibitions on the stability of competent state. A culture was made competent by treatment with CF. The competent culture was centrifuged and resuspended in four different kinds of media: in complete growth medium (1), in medium lacking glucose and sucrose (2), in medium lacking leucine (3), and in medium lacking leucine as well as glucose and sucrose (4). The number of transformable cells was determined after various periods of incubation at 30 C.*

glucose and sucrose. Third, the processes of "maintenance" and "decay" balance each other.

The titer of CF in tube 3 declined only slightly during the great drop in the number of competent cells. Thus, just as in the experiments illustrated in Fig. 15, the CF content of cells and their actual competence can be experimentally dissociated by inhibiting cellular protein synthesis.

Differential effects of metabolic inhibitors on the induction of competence versus the transformation process. The effect of metabolic inhibitions was tested on the bacteria during their interaction with the CF as well as after the completion of the induction process, i.e., in both phases 2 and 4 of the experiments (Table 1). Control experiments (*not shown*) have demonstrated that the various drugs exerted their characteristic inhibitory action (measured as inhibition of incorporation of the appropriate isotope tracer compounds) in an identical manner in both competent and incompetent cultures. Thus, the differences observed in the sensitivity of the "induction of competence" as compared to that of "transformation" reflect the relative importance of various cellular activities in these two processes. Although both "induction" and "transformation" were greatly inhibited by inhibition of energy metabolism (NaN_3, iodoacetate, energy source removal), induction was more sensitive (by several orders of magnitude) to inhibition of RNA and protein synthesis. In fact, the inhibition of transformation by inhibitors of protein synthesis must be partly due to the lability of the competent state under such conditions. Inhibition of DNA synthesis (up to 95%) had no effect on the induction or the transformation process.

DISCUSSION

In an attempt to learn about cellular activities which participate in various steps of genetic transformation, drug inhibition experiments were performed in three phases of genetic transformation. The effect of metabolic inhibitors was determined (i) in cells undergoing conversion to the competent state by CF, (ii) in the competent cells prior to reaction with transforming DNA, and (iii) during the process of DNA uptake by competent bacteria. All experiments started with uniformly incompetent cells growing exponentially at low *p*H.

The phases of the experiments were as follows: phase 1, incompetent cells growing in low *p*H medium; phase 2 (induction process), incompetent cell + CF → competent cell; phase 3 (maintenance of competence), competent cells, incubating; phase 4 (transformation process), competent cells + DNA → transformed cell.

The main conclusions of the experiments can be summarized as follows. (i) Cellular DNA synthesis can be drastically inhibited without effect on any of the three phases of the experiments. (ii) Inhibition of energy metabolism by glucose and sucrose starvation or by drugs inhibit both phases 2 and 4, whereas omission of glucose and sucrose from the growth medium has no effect in phase 3. (iii) Inhibitors of protein and RNA synthesis can cause dramatic inhibitions in both phases 2 and 3. It is not clear whether the less severe yet substantial inhibition of transformation (phase 4) by CAP, puromycin, and tryptazan represents a true inhibition of some step in DNA absorption or integration, or both, or is simply the consequence of the rapid destabilization of the competent state in the absence of protein synthesis.

TABLE 1. *Differential effects of metabolic inhibitors on various phases of genetic transformation*

Treatment (20 min)	Biosyntheses[a]			No. of transformants[b]	
	Protein	RNA	DNA	Drug treatment during induction of competence (incompetent cell + CF → competent cell)	Drug treatment during transformation (competent cell + DNA → transformed cell)
None (control)	100	100	100	100	100
No glutamine				10^{-3}	100
No leucine	5	5		1	60
No valine	5	6		0.5	
No arginine	8	5		1	
Chloramphenicol (100 μg/ml)	10	100	107	10^{-4}	33
Chloramphenicol (10 μg/ml)	20	100		10^{-4}	15
Puromycin (100 μg/ml)	10			10^{-4}	34
Tryptazan (10 μg/ml)	90			10^{-3}	66
No uracil	1	2	2	10^{-4}	120
Rifamycin (0.1 μg/ml)		16		10^{-3}	15
No thymidine (for 20 min)			5	300	
No thymidine (for 45 min)			5	30	
Fluorodeoxyuridine (10 μg/ml)	100		14	100	100
No glucose, sucrose				10^{-3}	0.9
NaN_3	59			10^{-4}	7
Iodoacetate	27			10^{-4}	0.8

[a] Measured as counts per minute per milliliter, expressed as per cent of control.
[b] Expressed as per cent of control.

The findings concerning requirement for protein synthesis require more detailed discussion. The induction process showed extreme sensitivity to inhibitory conditions as diverse as starvation of the bacteria of required amino acids or of uracil and different inhibitors such as CAP, puromycin, tryptazan, rifamycin, and actinomycin D (22). The simplest interpretation of these findings is that the establishment of the competent state requires the synthesis of a new protein.

The nature of these inhibitory effects is further specified by results of control experiments which showed that the various inhibitory conditions in the relatively small "doses" used had no effect on the viability of the cultures. The quick reversibility of the inhibitions also argues against the infliction of lasting nonspecific cellular damage. The possibility that the inhibitory conditions might cause a leakage of some cellular agent (nuclease or protease), which then could interfere with the induction process in a trivial way, is made unlikely by the finding that the inhibitions could be reversed by readdition of the limiting nutrient (e.g., valine) to the *same* medium in which the bacteria have been previously incubating in the absence of valine. The specificity of the requirements for protein synthesis during induction of competence is further emphasized by the extreme sensitivity of this process to inhibitors as compared to the relative insensitivity of the actual transformation process to the same drugs.

The inhibition of the induction of competence by the amino acid analogue, tryptazan, which does not block protein synthesis, suggests that, in this case, the effect of the drug is exerted by its incorporation into peptide bonds in place of tryptophan, resulting in a nonfunctional protein. This interpretation is in accord with the known mode of action of tryptazan in other biological systems. It seems, therefore, that the inhibitors of protein synthesis interfere with the acquisition of the competent state not merely by inhibiting a general increase of the cellular mass but rather by preventing de novo formation of a special class of protein molecules

The experiment with the valine- and uridine-limited cultures indicates that, at low growth rates, some cellular process rather than the concentration of CF becomes the limiting factor

in the overall rate of competence induction. It is suggested that this rate-limiting process is the synthesis of the same class of protein molecules cited in the preceding paragraph.

The experiments with the maintenance of competent state indicate that these proteins are highly unstable. Still other experiments suggest that they perform their function subsequent to the binding of CF by the cells.

Although practically nothing is known about the exact role these proteins play in genetic transformation, they seem to be required for the uptake and retention of DNA molecules because starvation of leucine and uridine was found to inhibit irreversible binding of radioactive DNA (22).

It has been known for some time that CAP can inhibit the spontaneous expression of competence during growth of pneumoccal cultures (7). Once evidence for the protein nature of the CF became available (22), it was tempting to interpret this inhibition simply as an interference with the endogenous production of CF during growth. Indeed, it was found that the autocatalytic production of pneumoccal CF (20) as well as the production of streptococcal CF by resting cell suspensions (R. Pakula and D. Iler, Can. J. Microbiol., *in press*) can be inhibited by CAP. Therefore, the finding that protein synthesis is still required for the establishment of the competent state, even when excess CF protein is supplied, was at first surprising. It seems quite clear now that inhibitors of protein synthesis added to a growing culture in the incompetent culture phase can inhibit the development of competence in two separate ways: (i) by preventing the endogenous synthesis of the CF protein, and (ii) by blocking the production of another protein which seems separate from the CF and which performs some function essential for the establishment of the competent state *after* the CF has associated with the bacteria.

The saturation kinetics of the induction process and the results of experiments with metabolic inhibitions suggest that the induction of the competent state occurs through a first reversible interaction between cell and CF which is followed by a second step requiring protein synthesis. The bacteria only become competent to undergo genetic transformation after step 2 is completed, and the maintenance of this competent condition requires uninterrupted synthesis of some proteins.

$$\text{Incompetent cell} + \text{CF} \rightleftharpoons \text{incompetent cell} - \text{CF}$$

$$\text{incompetent cell} - \text{CF} \xrightarrow[?]{\text{protein synthesis}} \text{competent cell}$$

There are several close similarities among the different transformation systems concerning metabolic requirements of genetic transformation. Inhibition of energy metabolism inhibits DNA uptake by *Haemophilus influenzae* (3, 18) as well as by *Bacillus subtilis* (23), and, extensive thymidine starvation of a thymidine-requiring *B. subtilis* auxotroph (2) does not seem to prevent development of competence. Furthermore, in each one of the major transformation systems, development of competence can be inhibited by inhibition of protein or of RNA synthesis in bacteria exposed to the competence "provoking" procedure [e.g., addition of CF to *Streptococci* (14), transfer to a special competence medium of *H. influenzae* (17), or nutritional stepdown in *B. subtilis* (23)].

Whether the protein(s) in question is a specific gene product directly involved with some step of genetic transformation (such as a DNA transport protein) is not clear at present. The synthesis of a specific DNA transport protein during the penetration of *Escherichia coli* cells by T5 bacteriophage has been suggested recently (11). A less specific alternative is equally plausible, however; namely, protein synthesis may be required in an indirect way, for instance to repair some hypothetical cellular damage (e.g., to plasma membrane) caused by the CF or by the other competence-provoking procedures. Clearly, a large number of specific possibilities could be considered within each one of these models.

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