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Development of Competence of *Haemophilus influenzae*

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ABSTRACT

SPENCER, HUGH T. (The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Md.), AND ROGER M. HERRIOTT. Development of competence of *Haemophilus influenzae*. J. Bacteriol. **90**:911-920. 1965.—A chemically defined nongrowth medium was developed for the induction of competence of *Haemophilus influenzae* by a stepdown procedure. Cells grown logarithmically in Heart Infusion Broth became competent after being transferred to a medium which consisted of amino acids, sodium fumarate, and inorganic salts. Chloramphenicol (2 μg/ml) or L-valine (1 μg/ml) in the nongrowth medium inhibited development of competence. The inhibitory action of L-valine was reversed by comparable concentrations of L-isoleucine. Kinetic studies of the development of competence showed a variable capacity of competent cells to take up deoxyribonucleic acid and reaffirmed earlier findings that competence was not transmissible in *H. influenzae*. Addition of nicotinamide adenine dinucleotide, thiamine, calcium pantothenate, uracil, and hypoxanthine to the medium for competence resulted in a minimal growth medium in which reduced levels of competence were developed.

The ability of some species of bacteria to take up extracellular deoxyribonucleic acid (DNA) from related strains and to integrate it into the recipient's genome has been termed competence (Thomas, 1955). Goodgal and Herriott (1961) reported the development of high levels of competence in *Haemophilus influenzae* Rd under conditions of reduced aeration in both Brain Heart Infusion and Levinthal broth. A medium of known constituents was developed (Talmadge and Herriott, 1960) which supported growth of cells, but such cells failed to develop appropriate levels of competence. The present report describes a nongrowth medium, designated M-II, for the induction of competence of *H. influenzae* by a stepdown from complex growth conditions to the simple medium. Cells grown in Heart Infusion Broth and then transferred into medium M-II developed high levels of competence during 3 hr of aeration. This system permitted study of the kinetics of the development of competence and some of the factors and conditions which induce the phenomenon.

MATERIALS AND METHODS

Test organism. The organism studied was *H. influenzae* Rd, originally supplied by Hattie E. Alexander of the Department of Pediatrics, College of Physicians and Surgeons, Columbia University. The classification as type Rd was kindly reaffirmed in 1963 by Grace Leidy, also of Columbia University.

Preparation of medium M-II for competence. The composition of medium M-II is given in Table 1; the medium was routinely made up on the day of use from five independent stock solutions. To prepare 1 liter of solution 1, the following components were added to 850 ml of distilled water: aspartic acid, 4.0 g; glutamic acid, 0.2 g; fumaric acid, 1.0 g; NaCl, 4.7 g; K_2HPO_4, 0.87 g; KH_2PO_4, 0.67 g; Tween 80 (Mann Research Laboratories, New York, N.Y.), 0.2 ml. The *p*H was adjusted to 7.0 with 1 N NaOH, and distilled water was added to make 1.000 ml. Solution 2 contained all the remaining amino acids in the medium except histidine (Table 1) concentrated 100-fold in 0.10 N HCl. The cystine and tyrosine in 100 ml of solution 2 were initially dissolved at 37 C in 10 ml of 1 N HCl, and the solution was immediately diluted to 0.1 N with distilled water. The remaining amino acids were added to solution 2 after it had been diluted to 0.1 N HCl. Solutions 3 and 4 contained 0.1 M $CaCl_2$ and 0.05 M $MgSO_4$, respectively. Solution 5 was prepared by adding 0.1 g of hemin and 0.1 g of histidine to 100 ml of 4% (v/v) 2,2′,2″-nitrilotriethyl alcohol, which was then heated for 10 min at 60 C. Solutions 1, 3, and 4 were sterilized by autoclaving; solution 2, by filtration through a Millipore disc of 0.22-μ porosity.

The medium was prepared by adding, in order,

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TABLE 1. *Composition of defined medium M-II for development of competence*

Component	Final concn
Essential compounds	
L-Aspartic acid	4,000 μg/ml*
L-Glutamic acid	200 μg/ml*
L-Cystine	4 μg/ml
L-Arginine or L-citrulline	6 μg/ml
Supplemental compounds	
Fumaric acid	1,000 μg/ml*
L-Phenylalanine	20 μg/ml
L-Tyrosine	10 μg/ml
L-Serine	30 μg/ml
L-Alanine	20 μg/ml
Hemin + histidine	10 μg/ml
Salts†	
Tween 80	0.02%
NaCl	0.08 M
$MgSO_4$	5×10^{-4} M
$CaCl_2$	1×10^{-3} M
K_2HPO_4-KH_2PO_4 (1/1)	0.01 M

* Neutralized with 1 N NaOH in preparation of the medium.

† Salts mixture (including Tween 80) shown by Cabrera-Juarez and Herriott (1963) to be optimal for the uptake of DNA by cells made competent in Brain Heart Infusion.

1 ml each of solutions 2, 3, 4, and 5 to 100 ml of solution 1. Final *p*H of the medium was 7.2 to 7.3.

The medium was routinely prepared with amino acids and fumaric acid purchased from Mann Research Laboratories (M.A. grade). Medium M-II, prepared with A-grade reagents from Calbiochem induced the same level of competence observed in media prepared with products from Mann Research Laboratories. Requirements for cystine and glutamate could not, however, be demonstrated with media in which the aspartic acid was supplied by Nutritional Biochemicals Corp., Cleveland, Ohio, lot no. 1778.

The minimal growth medium M-IIg was prepared by adding nicotinamide adenine dinucleotide (NAD), thiamine, calcium pantothenate, uracil, and hypoxanthine to complete M-II at respective levels of 2, 2, 2, 20, and 20 μg/ml.

Routine incubation in M-II and exposure to DNA. Studies of the development of competence in M-II were made with cells cultured in 2.5% Heart Infusion (Difco). At the beginning of a typical experiment, 50 ml of Heart Infusion were inoculated with 2 ml of Rd cells (at 10^9 per milliliter) which had been stored at −60 C in Heart Infusion containing 17.5% glycerol. The culture was agitated gently at 37 C in a 500-ml Erlenmeyer flask until a density of 10^9 cells per milliliter was reached, as judged by absorbance in a Coleman junior spectrophotometer at 650 mμ. Cells were then harvested by centrifugation at room temperature at 1,600 × *g* for 7 to 10 min in a Clinical International centrifuge. The cells were centrifuged twice and suspended in the initial volume with the salts of M-II, and were finally concentrated 10-fold to give 10^{10} cells per milliliter. The concentrated preparations were immediately diluted into 2.0 ml of M-II in culture tubes (18 by 150 mm) to give $7 \times 10^8 \pm 10\%$ cells per milliliter. The cultures were then agitated for 180 min at 37 C. S DNA (DNA isolated from cells resistant to 2,000 μg of streptomycin per ml) was added at the end of the 180-min period at 1 μg/ml along with 10 μg/ml of L-valine, which stopped any further development of competence. Exposure to DNA was terminated in 40 min by the addition of deoxyribonuclease at a concentration of 5 μg/ml.

The reproducibility of the development of competence in medium M-II is shown in Table 2, in which the statistics are compiled for 11 independent experiments conducted over a 6-month period. The coefficients of variation are considered small in view of the experimental error inherent in determinations based on serial dilutions.

Determination of optimal cell concentration. The above procedure was necessarily modified for the experiment given in Fig. 1. In that instance, cells harvested from HI were resuspended in M-II at the indicated concentrations and aerated for 180 min. Chloramphenicol (5 μg/ml) was then added to each culture, and the populations were diluted according to their density into M-II plus chloramphenicol to give about 10^8 cells per milliliter. S DNA (1 μg/ml) was added to the dilute populations (0.6×10^8 to 1.2×10^8 cells per milliliter) for a 40-min exposure terminated by the addition of deoxyribonuclease.

Return of competent cells to growth conditions. Figure 2 shows the effect upon competence of returning cells, prepared in medium M-II, to conditions of growth at 37 C in Heart Infusion and M-IIg. Cells which had just completed 180 min of incubation in M-II were diluted 10-fold into the growth media, and the cultures were immediately divided into a series of tubes containing 1.0 ml each. Individual tubes were selected for the measurement of competence and viable titers at 5-min intervals for the Heart Infusion culture and at 20-min intervals for the M-IIg culture. In both cases, S DNA (1 μg/ml) was added to the selected tube, and the contents were diluted exactly 5 min later into diluent containing deoxyribonuclease (5 μg/ml) and 5×10^{-3} M $MgSO_4$. The maximal periods of incubation in Heart Infusion were 85 min and in M-IIg, 180 min.

The medium for competence, M-II, and the minimal growth medium, M-IIg, were supplemented in this experiment with 4 μg of leucine per ml and 1 μg of valine per ml.

Lack of transmissibility of competence in M-II. An experiment dealing with the possibility of competence being transmissible in *H. influenzae* Rd is given in Fig. 6. Cells resistant to streptomycin (S) in one case and novobiocin (N) in the other were inoculated into Heart Infusion with inocula of equal cell density and identical treatment except that the S cells were inoculated 60 min before the

TABLE 2. *Statistics compiled for the development of competence observed in medium M-II in 11 independent experiments conducted over a 6-month period*

Determination	Viable cells/ml × 10^{-8}		Transformations* per ml × 10^{-7}	Per cent transformation
	Initial	Final		
Mean	6.97	9.64	3.45	3.58
Standard deviation	0.83	1.36	0.76	0.49
Coefficient of variation	12%	14%	22%	14%

* Transformants per milliliter to streptomycin resistance observed after a 40-min exposure of cells incubated for 180 min in M-II to an excess of DNA isolated from a strain resistant to 2,000 μg of streptomycin per ml.

N cells. S cells were subsequently harvested from the broth when the culture density reached 10^9 cells per milliliter, and were suspended in M-II at 7×10^8 cells per milliliter and incubated at 37 C as usual. The N cells attained a density of 10^9 per milliliter 60 min later and were similarly resuspended in M-II at 7×10^8 per milliliter with the same washing procedure used for S. The two cultures were then mixed in equal parts; 2-ml samples were placed in a series of tubes, and the incubation at 37 C was continued. DNA from erythromycin-resistant cells (E) and L-valine were added simultaneously to individual tubes at selected times to give levels of 1 and 10 μg/ml, respectively. Exposure to DNA was terminated 40 min later by the addition of deoxyribonuclease. The kinetics of the development of competence in the S and N populations were followed by titering transformants to E on agar containing 15 μg of erythromycin per ml plus 250 μg of streptomycin per ml in one case and 15 μg of erythromycin per ml plus 25 μg of novobicin per ml in the other.

Transforming DNA. The transforming DNA used was isolated by methods previously described (Goodgal and Herriott, 1961).

Transformation assay. Transformants to antibiotic resistance were assayed by the overlay technique. Transformants to citrulline utilization (Table 4) were assayed by plating appropriate dilutions of DNA-cell reaction mixtures directly into the minimal medium (M-IIg) with 1.25% agar and with citrulline in the place of arginine and uracil.

Each experiment concerning the development of competence in M-II was accompanied by appropriate controls for the detection of chance contamination.

RESULTS

Stepdown induction of competence. Work had begun on defining conditions for the development of competence when Stuy (1962) and Leidy, Jaffee, and Alexander (1962) reported an enhancement of competence in *H. influenzae* by a stepdown procedure. Cells at a density of 3×10^9 per milliliter were treated by these workers with an aerobic-nonaerobic cycle in Levinthal broth and then diluted 10^4-fold into 1% aspartate in 0.01 M phosphate-buffered saline. Transformability in these cultures increased 15- to 20-fold after 60 min of aeration in the aspartate-phosphate-saline solution. An extension of this work led to the nongrowth medium M-II, which induces high levels of competence in log-phase cells grown in Heart Infusion Broth. Continuous aeration in M-II by agitation was found to be superior to the standing period of 105 min. preceding agitation, as recommended earlier (Spencer and Herriott, 1963).

Cells harvested at any time during logarithmic growth in Heart Infusion were capable of becoming competent in M-II, although the concentration of cells in the medium was found to be critical. An optimal initial cell concentration was observed to lie between 5×10^8 and 1×10^9 cells per milliliter (Fig. 1). A 50% decrease in viable cells was also observed upon incubation for 3 hr in M-II at concentrations below the optimum. Cells incubated in the medium at the optimum and above remained fully viable.

Nutritional requirements for competence. The simplest medium giving a reasonable level of competence consisted of the four essential amino acids listed in Table 1, together with 0.1% fumaric acid (Table 3). Deletion of any one of these amino acids from the complete medium resulted in its failure to promote competence and in a 50 to 75% loss in viable cells during incubation for 3 hr. An analogous result was obtained by the addition of chloramphenicol to the medium in a concentration of 2 μg/ml.

The addition of hypoxanthine, uracil, thiamine, calcium pantothenate, and NAD to M-II formed a minimal growth medium which was designated M-IIg. The organism has been subcultured in the minimal medium and forms colonies when plated in it with 1.25% agar. The four amino acids found essential for the development of competence were also found necessary for growth in M-IIg, including the same high level of aspartate.

Neither fumarate not the supplemental amino acids were necessary for growth.

Further study of the arginine requirement for the development of competence in M-II revealed that it could be replaced by citrulline, but not by ornithine. In the case of growth in the minimal medium M-IIg, citrulline replaced not only the arginine, but also the uracil requirement, possibly by donating its carbamyl group to the uracil pathway via ureidosuccinic acid (Schulman and Badger, 1954). The mechanism of this conversion is presently speculative in *H. influenzae*, although a mutant has been isolated from ultraviolet-treated cells which fails to grow on citrulline unless uracil is added. The mutant also fails to become competent in M-II in the absence of uracil, whereas the wild type could become competent on either arginine or citrulline in the absence of uracil (Table 4). The experiments given in this report were all conducted with M-II prepared with citrulline.

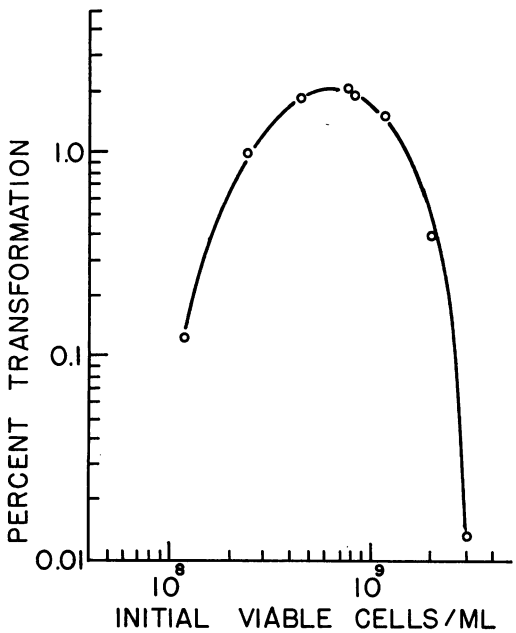

FIG. 1. *Per cent transformation to streptomycin resistance observed in cultures incubated at different cell concentrations for 180 min in medium M-II.*

Growth and competence. Discovery of the minimal growth medium M-IIg afforded an opportunity to assess more critically the influence of growth conditions on the development of competence. Two types of experiments were performed. One in which the development of competence in M-IIg was assessed (Table 5), and another in which the loss of competence was followed after the return of competent cells to growth conditions (Fig. 2).

The maximal cell density obtainable in M-IIg was found to be in the range of 10^9 cells per milliliter. It thus seemed probable that, if growth were adverse to competence, cells transferred from Heart Infusion into M-IIg at the usual concentration of 7×10^8 cells per milliliter would likely become competent, since this level of cells is near the maximal density supported by the medium. On the other hand, cells resuspended in M-IIg at lesser concentrations should exhibit

TABLE 3. *Nutritional requirements for competence in M-II*

Medium	Viable cells/ml	Transformants/ml*	Per cent transformants
Basic medium†	7.3×10^8	2.5×10^4	0.3×10^{-2}
Basic medium			
+ Hemin	5.7×10^8	1.5×10^5	0.3×10^{-1}
+ Amino acids‡	8.9×10^8	2.7×10^6	0.3
+ Fumarate	9.8×10^8	1.7×10^7	1.7
Complete M-II	1.0×10^9	3.1×10^7	3.1
Complete M-II			
− Aspartate	3.3×10^8	1.1×10^3	0.3×10^{-3}
− Glutamate	1.8×10^8	1	10^{-6}
− Cystine	3.4×10^8	1.3×10^3	0.4×10^{-4}
− Arginine	2.4×10^8	2×10^1	0.8×10^{-5}

* Transformants per milliliter to streptomycin resistance observed after a 40-min exposure of cells in the test media to an excess of DNA isolated from a strain resistant to 2,000 μg of streptomycin per ml. Deletion of individual organic components from the medium had no effect on the uptake of DNA by cells already made competent in complete M-II.

† Four essential amino acids (aspartate, glutamate, cystine, and arginine) and salts.

‡ Tyrosine, phenylalanine, serine, and alanine.

growth and be less competent at the end of the 3-hr incubation. These predictions were borne out by the duplicate experiments presented in Table 5. The development of competence in M-IIg with initial inocula of 6×10^8 to 8×10^8 cells per milliliter was two- to three-fold less than in M-II at comparable cell concentrations and as much as 80-fold less in cultures in which more extensive growth was permitted.

A strict interpretation of these data is not possible in view of the fact that dilute populations of cells do not become as competent in M-II as do cultures initiated in the range of the cell concentration optimum, shown in Fig. 1. However, the initial cell concentrations chosen for M-IIg in the experiments given in Table 5 were not sufficiently removed from the optimum to account for the lower levels of competence observed after 3 hr of incubation. This is especially true since the cell number increased during the incubation up to and slightly beyond the optimum.

An exponential loss in competence concurrent with an increase in cell number was also observed in cells made competent in M-II and resuspended in either Heart Infusion Broth or the minimal growth medium M-IIg (Fig. 2). The drop in competence per generation was essentially the same in both media, although the generation time in Heart Infusion (30 min) was one-third of that observed in the minimal medium. Taken together, these results confirm earlier observations concerning the loss of competence in growth media (Goodgal and Herriott, 1961) and suggest that the development of competence and growth are competitive in *H. influenzae* and may be mutually exclusive processes.

Inhibition of the development of competence by L-*valine.* Experiments with amino acid requirements showed L-valine to be a strong inhibitor of the development of competence in M-II (Fig. 3). An investigation of this phenomenon resulted in the following observations: (i) 1.0 μg/ml of L-valine reduced the development of competence to less than 0.01% of the control; (ii) L-valine added to competent cells did not prevent DNA uptake; (iii) it has no effect on the viability of the cells; and (iv) the inhibition by L-valine was completely reversed by either L-isoleucine or

TABLE 4. *Pyrimidine requirement for development of competence*

Medium (M-II)* prepared with	Per cent transformation to streptomycin resistance†	
	Mutant	Wild type‡
Arginine	0.007	1.5
Citrulline	0.011	2.3
Arginine + uracil	2.6	2.6
Citrulline + uracil	2.9	2.2

* All components listed present at levels of 10 μg/ml.

† Conditions of DNA exposure were the same as in Table 3.

‡ Transformant to citrulline utilization derived from the mutant. A transformation frequency to citrulline utilization of 0.5% was obtained in the above experiment with cells made competent in the citrulline plus uracil medium.

TABLE 5. *Effect on the development of competence of adding growth factors* to M-II*

Expt	Medium	Viable cells/ml		Generations	Transformants/ml†	Per cent transformation
		Initial	Final			
1	M-II	7.8×10^8	1.3×10^9	0.8	5.4×10^7	4.0
	M-IIg	7.0×10^8	1.8×10^9	1.4	3.2×10^7	1.8
		3.4×10^8	1.4×10^9	2.1	1.4×10^7	1.0
		1.9×10^8	2.3×10^9	3.6	1.3×10^6	0.05
2	M-II	6.0×10^8	1.0×10^9	0.8	3.7×10^7	3.7
	M-IIg	7.8×10^8	1.6×10^9	1.1	1.9×10^7	1.2
		4.1×10^8	1.4×10^9	1.8	8.2×10^6	0.6
		2.0×10^8	9.5×10^8	2.3	8.0×10^5	0.09
		1.1×10^8	5.1×10^8	2.2	7.8×10^5	0.15

* Hypoxanthine, uracil, calcium pantothenate, thiamine, and NAD at 20, 20, 2, 2, and 2 μg/ml, respectively. Addition of the growth factors resulted in the minimal growth medium, M-IIg.

† Transformants per milliliter to streptomycin resistance observed after a 40-min exposure of the cells in M-II at the above densities to an excess of DNA isolated from a stran resistant to 2,000 μg of streptomycin per ml.

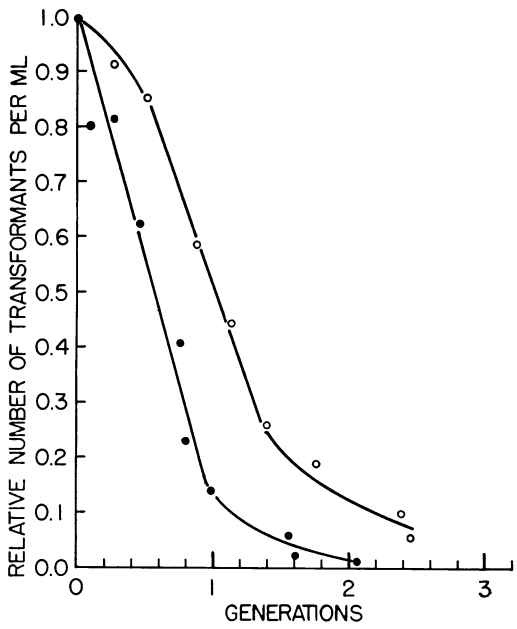

FIG. 2. *Loss of transformability as a function of growth at 37 C after a step up from the medium for competence to the minimal growth medium and to Heart Infusion Broth. Symbols:* ○ = *relative number of transformants to streptomycin resistance observed with a 5-min pulse of DNA given to cells transferred to the minimal growth medium after 180 min of incubation in the medium for competence;* ● = *relative number of transformants to streptomycin resistance observed with a 5-min pulse of DNA given to cells transferred to Heart Infusion Broth after 180 min of incubation in the medium for competence.*

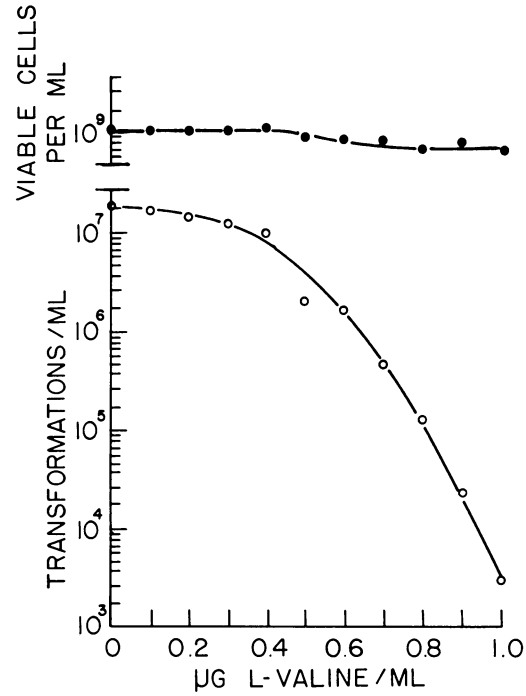

FIG. 3. *Influence of increasing concentrations of* L*-valine on the development of competence in medium M-II as measured by transformants to streptomycin resistance. Symbols:* ● = *viable cells per milliliter;* ○ = *transformants per milliliter.*

L-leucine at comparable concentrations (Table 6). Low levels of L-valine also inhibited growth in the minimal medium, and this inhibition was similarly reversed by either L-isoleucine or L-leucine.

The inhibitory action of L-valine was utilized in assessing the kinetics of the development of competence in M-II by adding 10 μg/ml of the amino acid at selected times to stop further development. The addition of chloramphenicol or the removal of essential amino acids also blocked the development of competence at specific times, but these methods were judged less suitable because their use led to a loss of viability.

Kinetics of the development of competent cells. The absolute number of competent cells can be estimated in a given culture from the relationship of single to double transformants after exposure to a mixture of transforming DNA (Goodgal and Herriott, 1961.

$$\frac{N_A \times N_B}{N_{AB}} = C_0 \qquad (1)$$

N_A and N_B are the observed number of single transformants to independent markers A and B, and N_{AB} is the number of double transformants resulting from the random incorporation of both markers into a recipient cell. C_0 is the calculated number of competent cells. The two independent markers chosen for the following experiments were resistance to 2,000 μg of streptomycin sulfate per ml (S) and to 15 μg of erythromycin lactobionate per ml (E).

The course of the development of competence in M-II is given in Fig. 4. The number of competent cells in the initial inoculum as determined by equation 1 (0.2% of the total viable cells) was characteristic of cells grown in Heart Infusion, and remained essentially constant for the first 30 min of incubation in M-II. Competence began to develop between 30 and 60 min and rose rapidly, reaching a plateau by 150 min. A characteristically rapid development of competence has previously been described in *Diplococcus pneumoniae* (Hotchkiss, 1954; Thomas, 1955), *Streptococcus* sp. (Perry and Slade, 1963), *Bacillus subtilis* (Nester, 1964), and *H. influenzae* (Goodgal and Herriott, 1961).

The discrepancy in Fig. 4 between the plateau

TABLE 6. *Effect of leucine and isoleucine on the inhibition by valine of the development of competence in medium M-II*

Isoleucine	Leucine	Valine	Viable cells/ml	Transformants/ml*	Per cent transformation
mμmoles/ml	*mμmoles/ml*	*mμmoles/ml*			
0	0	0	9.5×10^8	3.1×10^7	3.3
0	0	20	5.5×10^8	5.8×10^3	0.001
0	0	9	9.9×10^8	6.9×10^4	0.007
20	0	0	9.9×10^8	3.6×10^7	3.6
10	0	20	1.0×10^9	4.0×10^7	4.0
20	0	20	1.0×10^9	3.4×10^7	3.4
0	30	0	1.5×10^9	6.2×10^7	4.1
0	30	9	1.3×10^9	7.1×10^7	5.7

* Conditions of DNA exposure were the same as in Table 3.

levels of viable cells and the calculated number of competent cells was obtained repeatedly, but is presently without a proven explanation. The suggestion by Nester and Stocker (1963) that such calculations be based on the number of nuclei per milliliter, rather than on viable titers, may be relevant here, since *H. influenzae* appears to be binucleated (Berns, 1964). It was also observed in other experiments that cell titers based on direct microscopic counts agreed more closely with calculated values for competent cells per milliliter than did viable counts.

Varying levels of competence in individual cells. The next question considered was whether competent cells appearing in the medium possessed their full capacity to absorb DNA at the time of their initial appearance. This question was answered by examining the ratio of double to single transformants among cells exposed to a mixture of unlinked transforming DNA preparations during the period of the development of competence. The ratios N_{SE}/N_S and N_{SE}/N_E should remain constant throughout the course of development if the full capacity of a competent cell to incorporate DNA appears suddenly, i.e., if competence is an all or none phenomenon. If, on the other hand, the capacity of a cell to incorporate DNA increases during the development, the ratios N_{SE}/N_E and N_{SE}/N_S should also increase.

The ratios of double to single transformants for the experiment given in Fig. 4 are plotted against time in Fig. 5. The drop in the ratios at 60 min was obtained repeatedly, and suggests that the competent cells which developed between 30 and 60 min incorporated less DNA per cell than those few which were originally present. After 60 min the trend reversed and the ratio increased 10-fold to a maximum in 180 min.

The above kinetic data indicate that during the development of competence there is an increase in the absolute number of competent cells and an increase in the quantity of DNA that an individual competent cell can incorporate.

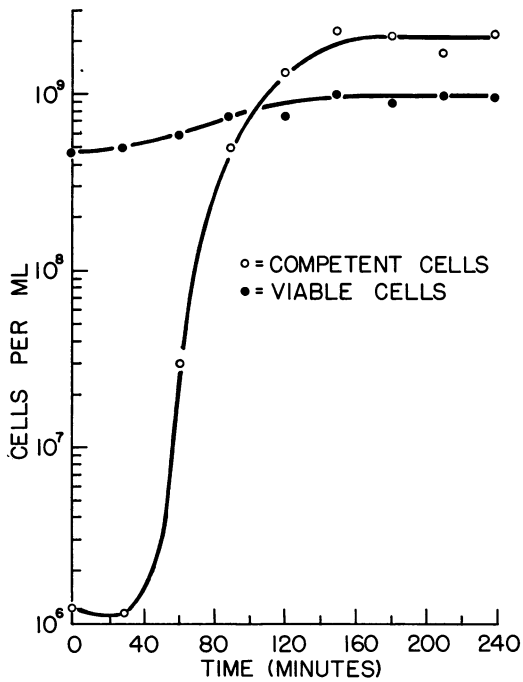

FIG. 4. *Calculated number of competent cells developing in M-II medium as a function of time of incubation.*

Lack of transmissibility of competence in H. influenzae. The possibility that competent cells or their supernatant fluids could accelerate the development of competence in noncompetent cultures was previously considered in this laboratory (Goodgal and Herriott, 1961). Evidence was obtained which suggested that competence could be lost and regained through the removal and replacement of 10^{-4} M calcium ion, but these results were not considered as evidence for transmissibility.

In other systems, several investigators (Pakula and Walczak, 1963; Tomasz and Hotchkiss,

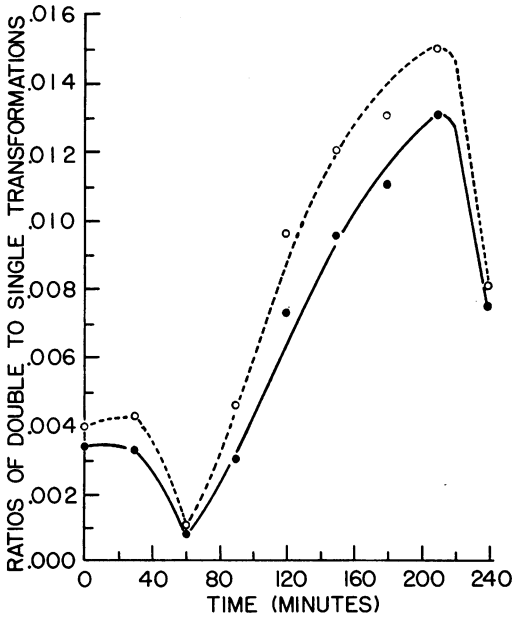

FIG. 5. *Ratio of double to single transformants as a function of time of incubation in medium M-II, a measure of the development of various levels of competence in individual cells. Symbols:* ○ = *ratio of cells resistant to streptomycin and erythromycin to cells resistant to streptomycin;* ● = *ratio of cells resistant to streptomycin and erythromycin to cells resistant to erythromycin.*

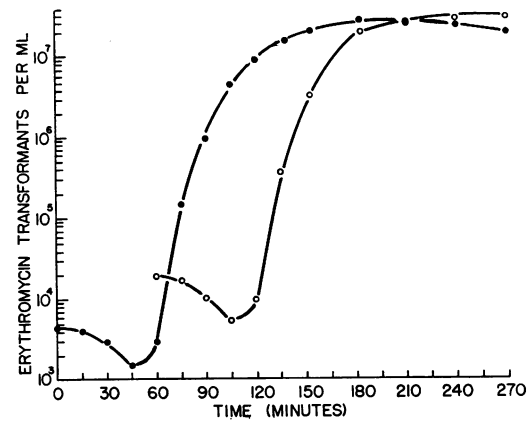

FIG. 6. *Test for transmissibility of competence. Cells resistant to streptomycin were mixed after 60 min of incubation in medium M-II with an equal proportion of noncompetent novobiocin-resistant cells. The development of competence in both the initial (streptomycin-resistant) and mixed (streptomycin- and novobiocin-resistant) cultures wcs followed by transformation to erythromycin resistance, which is plotted as a function of time of incubation in medium M-II. Symbols:* ● = *transformants to erythromycin resistance observed per milliliter of mixed culture after screening on agar containing 250 μg of streptomycin per ml and 15 μg of erythromycin per ml;* ○ = *transformants to erythromycin resistance observed per milliliter of mixed culture after screening on agar containing 25 μg of novobiocin per ml and 15 μg of erythromycin per ml.*

1964; Felkner and Wyss, 1964) have recently reported that filtrates of competent cells accelerate the rate of formation of competence in noncompetent cells. In a re-examination of the problem in *H. influenzae*, two cell lines resistant to different antibiotics (streptomycin [S] and novobiocin [N]), were made competent in M-II in the same culture tube. The culture carrying the novobiocin marker was added 1 hr after the streptomycin-resistant culture was started, and in such a way as not to alter the total cell concentration. Assays of competence were made on samples of the suspension by simultaneously adding L-valine and an excess of DNA carrying resistance to erythromycin (E), and then screening for transformants resistant to both S and E (initial population) and to N and E (test population). The results of this experiment are given in Fig. 6, and show clearly that the development of competence in the second population was not perceptibly influenced by the first, which became fully competent during the time-lag period of the second culture. Control experiments showed the kinetics of the development of competence in N populations to be identical to that in Fig. 6 when mixed with S cells at zero-time and when incubated alone.

These data do not exclude transmissibility of competence in *H. influenzae*, but, if it is found, it will likely be under different conditions or may apply to only a small fraction of the cells.

DISCUSSION

The conditions described for the induction of competence in *H. influenzae* by a stepdown from Heart Infusion Broth to the medium for competence (M-II) may be unique for the strain studied. The amino acids required for the development of competence are the same as those required for growth in the minimal medium (M-IIg), and probably have no special relation to transformability other than support of protein synthesis. Unrecognized differences in nutritional requirements among substrains of *H. influenzae* Rd might then be reflected in their inability to become competent in M-II without modification of the medium. Such was the case with the auxotroph which failed to grow in M-IIg on citrulline in the absence of uracil and subsequently failed to become competent in M-II in the absence of uracil (Table 4).

The medium M-II is clearly an optimal en-

vironment for the induction of competence in *H. influenzae*, but the data presented have not elucidated the inducing mechanism. However, the results given in Table 5 and Fig. 2 suggest that the cessation of duplication is an important feature of the induction. This is especially true in view of the development of reasonable levels of competence in M-IIg at cell concentrations approximating the maximal level of growth supported by the medium (Table 5). Apparently, just the presence of growth factors in the medium is not sufficient to retard the development of competence. The fact that the drop in competence per generation is essentially the same in two completely different growth media (M-IIg and Heart Infusion, Fig. 2) supports the contention that suppression of duplication is crucial to competence in *H. influenzae*.

The relationship between suppression of growth and the development of competence may explain why competent *H. influenzae* cells appear under such a variety of culture conditions, since any environment which is adverse to growth but permits protein synthesis might promote competence. Four sets of culture conditions for the induction of competence which agree with this proposal have been documented in the case of *H. influenzae*: (i) in the stationary phase of growth in complex media; (ii) after the interruption of exponential growth in complex media by reduced aeration; (iii) incubation in dilute broth or in the aspartate medium of Leidy et al. (1962); and (iv) incubation in medium M-II. The results in Fig. 6 suggest that, at least in the case of M-II, induction takes place via some internal cellular change rather than by an exogenous transmissible factor.

The bulk of evidence in other transformable organisms also indicates that selective culture conditions and protein synthesis are essential for competence, but evidence showing the induced synthesis of an enzymatic or active site for DNA absorption has not yet been found. The studies of Young, Spizizen, and Crawford (1963) with *B. subtilis* and of Ephrussi-Taylor and Freed (1964) with *D. pneumoniae* indeed suggest that the protein synthesized could be an autolytic enzyme which acts upon the cell wall to form a partial protoplast, thereby permitting the uptake of DNA. The uptake of phage DNA by spheroplasts of *Escherichia coli* (Guthrie and Sinsheimer, 1960; Sekiguchi, Taketo, and Takagi, 1960; Meyer et al., 1961) and of *Shigella paradysenteriae* (Wahl, Huppert, and Emerique-Blum, 1960) agrees with the suggestion that competent cells may possess some features of protoplasts.

Barnhart and Herriott (1963) concluded from an investigation of factors influencing the uptake of P^{32}-labeled DNA by competent *H. influenzae* cells that DNA penetrated into the cells via a highly ordered and specific mechanism. Stuy and Stern (1964) concluded from the inhibition of DNA uptake by dinitrophenol that uptake of DNA by competent *H. influenzae* cells is enzymatic and energy-dependent. Consideration of competent *H. influenzae* simply as protoplasts does not explain the evidence suggesting that an active structure mediates DNA uptake. However, a possible deterioration of the cell surface under conditions found favorable to the development of competence could easily expose specific structures which normally remain hidden. If these structures had normal functions involving the cell's genome, they might well be equipped to draw the extracellular transforming DNA into the cell once contact is made.

The appearance of competent *H. influenzae* cells via directed synthesis of active sites as compared with the exposure of already existing structures seems equally possible. The present studies do not permit a choice between these two generalities, but do provide the essential background for a broader attack on the unique problem of how cells become capable of taking up extracellular DNA.

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