Studies on Transformations of *Hemophilus influenzae*

I. Competence

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ABSTRACT A procedure has been developed for obtaining *Hemophilus influenzae* of such competence that 1 to 10 per cent transform to any of several genetic factors by utilizing a period of aerobic growth followed by a non-aerobic period. Differences in levels of competence were not due to differences in genetic background. Competence was due to at least one factor intrinsic to the cell or site on the cell and was not transferable to non-competent cells. Competence was affected by salt concentration, pH, and temperature. Washing competent cells reduces their ability to transform, but not their capacity to bind DNA reversibly. The irreversible step could be restored with little or no accompanying growth. These facts suggest that reversible and irreversible binding represent separate biochemical steps. DNA initiates a reaction in cells leading to a loss of competence. In the absence of DNA the cells remain competent for at least an hour. Competence correlates quantitatively with predictability of multiple transformations. The observed and calculated values of multiple transformations are in closer agreement, the higher the frequency of transformation for single markers. The correction needed to bring the two figures into agreement is a measure of the fraction of non-competent cells.

INTRODUCTION

Competence has usually been defined as a capacity of the cell to undergo transformation. The term has been used in a relative sense to denote populations of cells which produce either a high or low level of transformation for a particular characteristic. The transformation process involves a number of steps, any one of which may vary, and therefore affect the competence of a population. However, in this and subsequent papers competence is defined in terms of the ability of the recipient cell to take up DNA irreversibly (and subsequently undergo transformation). In view of the fact that additional transforming systems are appearing and an increasingly large number of viral nucleic acids are proving to be infective, the value of understanding the mecha-

nism of competence takes on added significance. The competence of a cell is an essential phase in the infection or parasitism and genetic modification of cells by nucleic acids.

In *Hemophilus influenzae* not all cells undergo transformation to a given characteristic. The frequency of transformation may vary from 0 to 10 per cent over a span of as much as 6 logs with a reproducibility of ±15 per cent. In this publication the development of competence by receptor cells will be considered along with those conditions or agents which affect the ability of cells to undergo transformation in the presence of active DNA preparations.

MATERIALS AND METHODS

Many of the procedures used in the experiments have been modified from the excellent original procedures worked out by Alexander and Leidy (1). Our methods and modifications are presented in some detail below.

Media and Diluents The medium in which *H. influenzae* cells grew best in our hands is a modified Levinthal stock, hereafter designated as Elev broth. This was made by mixing equal quantities of Levinthal stock and 3 per cent Eugonbroth (Baltimore Biological Laboratories) followed by the addition of enough diphosphopyridine nucleotide (DPN) to make the concentration 2 μg/ml.

The Levinthal stock and Eugonbroth were bottled in 50 ml quantities and stored after being autoclaved.

Plating medium consisted of one part agar mixture and one part Levinthal stock + 2 μg/ml DPN. The agar mixture was made by dissolving and autoclaving 5 gm of agar and 6 gm Eugonbroth in 210 ml of H_2O. For pour plates we used 15 to 20 ml of media per plate and for agar-layer plates, two 10 ml layers were used. The agar medium may be kept several days at 45°C without deterioration, but once hardened it should not be remelted. Melted medium should be cooled to 37–40°C before pouring plates in order to avoid inactivation of *Hemophilus* cells. As a diluent for cells 1.5 per cent Eugonbroth was used, and for transforming factor (T.F.) citrate-saline (0.15 M NaCl + 0.014 M Na_3 citrate) was the diluent.

Growth of Cells *H. influenzae* cells were usually grown by inoculating 3 ml Elev broth containing 2 μg DPN/ml in a side-arm test tube at 35–37°C. The generation time was about 30 minutes. The turbidity at 650 mμ compared to a solvent control provided a simple method of determining the total number of cells present. (See Table I for the relationship between turbidity and viable cell count.) For log phase cells up to an optical density of 0.30, the relationship of turbidity to viable cells varied considerably, depending on the rate of shaking or aeration.

For growing larger quantities of cells, Erlenmeyer flasks containing an inch or two of medium were inoculated and kept in motion on a rotating shaker.

For purposes of concentrating *H. influenzae* cells or removing them from solution, cells were centrifuged for 5 minutes at 2000 × *g*.

Preparation of Competent Cells In the early phases of this work, competent cells were made according to the method of Alexander and Leidy (1). With slight modifi-

cation, this method was found to give results which were similar to those presented by Alexander and Leidy. More competent cells (frequency of 1 to 5 per cent) could be obtained, however, by following the aerobic growth with a period of non-aerobic incubation. The cultures were merely allowed to stand at 37°C for 90 minutes. Both these procedures will be discussed under the experimental results.

Preparation of Transforming DNA DNA was prepared from a fully grown culture of *H. influenzae*. The details of the procedure were conditioned somewhat by the quantities of DNA desired. For small quantities of DNA, 3 ml of cells were grown in test tubes and lysed with 0.1 per cent desoxycholate or by the addition of alkali at 37°C to pH 11. As a routine, 3 ml of cells were grown in Elev broth to a turbidity of between 0.3 and 0.6 in the Coleman Jr. spectrophotometer at 650 mμ (from 5×10^9 to 1.2×10^{10}), then 0.2 ml of 1 N NaOH was added, the cells shaken for 20 seconds or so, then the preparation was neutralized with 1 N HCl. The crude lysate was then heated at 60°C for 10 minutes to destroy any remaining cells. Such preparations were stored in the refrigerator or deep freeze for periods extending over more than a year without appreciable loss in activity. The yields of DNA obtained by this procedure, however, were inferior to those obtained when cells were centrifuged and resuspended in citrate-saline (0.15 M saline, 0.05 M citrate) and then lysed with NaOH. The pH of the solutions was determined by means of a Beckman pH meter with high pH electrodes.

For preparations of purified DNA in quantities of 10 to 200 mg, a procedure essentially similar to that of Hotchkiss (2) was used. The steps followed were: (*a*) Growth of cells in Elev media or a complex media, (*b*) centrifugation of the cells and resuspension in citrate-saline, (*c*) lysis of the cells with desoxycholate at pH 9–10 or NaOH at pH 11, (*d*) addition of NaCl to a concentration of 2 M and removal of protein and other materials by repeated treatment according to Sevag (3) with chloroform and octanol, (*e*) centrifugation of the mixture and removal of the supernatant, which is then precipitated from 50 to 75 per cent ethanol and resuspended in citrate-saline, (*f*) the addition of 2 to 5 μg/ml of RNAase to digest RNA, (*g*) another alcohol precipitation and dissolution of the precipitate, after which the soluble DNA is freed of extraneous particles by high speed centrifugation. The detailed description of a 170 mg batch of DNA is given below.

Three ml of 1.5×10^9 cells were seeded into 1.5 liters of Elev media and grown overnight with vigorous shaking to produce a heavy growth. This 1.5 liter culture was added to 12 liters of Difco brain heart infusion broth to which was added 38 mg of hemin (11) and 18 mg of DPN, plus 400 ml of Levinthal stock. The temperature of the bottle was raised to 37°C before the addition of the cells and a suction pump attached to the bottle in such a way as to produce vigorous aeration through a viscose sponge attached to glass tubing at the bottom of the bottle. It is important to maintain this vigorous aeration if possible, throughout the growth of the culture, which is maintained at 37°C. Dow-Corning silicone anti-foam (from Dow-Corning Co., Midland, Michigan) was used to reduce frothing. After 5 hours' growth the cells had reached a concentration of 10^{10}/ml and were centrifuged in a Sharples centrifuge and resuspended in 400 ml citrate-saline. The residual liquid was poured out of the centrifuge bowl, and only the well packed cells were used.

The cells were lysed with 20 ml of 1 N NaOH, the pH rising to 10.65, and neutralized with 20 ml of 1 N HCl to a pH of 8.0. The NaOH and HCl were added with vigorous stirring in order to prevent local concentrations of alkali and acid. 55 gm of solid NaCl was added, dissolved, and followed by 450 ml of chloroform-octanol (8:1). The mixture was divided into two flasks for convenience in order to facilitate shaking overnight on a wrist-action shaker.

The agitated mixture was then centrifuged in plastic containers at top speed in the International centrifuge, model PR-2, and the supernatant decanted. The colored supernatant was mixed with 1 volume of 95 per cent ethanol and the precipitate separated and resuspended in 200 ml of 2 M NaCl + 0.05 M Na_3 citrate.

Two mg of RNAase were added to the redissolved DNA suspension and the mixture allowed to shake until the material was dissolved (overnight). An additional 3 mg of RNAase was added and the solution maintained at room temperature for 1 hour or more. 200 ml of chloroform-octanol was added and after shaking the mixture overnight, it was centrifuged in the International PR-2 centrifuge, the supernatant decanted, and the nucleic acid precipitated with 1.5 volumes of alcohol, after which it was resuspended in 200 ml of citrate-saline (0.05 M citrate + 2 M NaCl). After the DNA had gone into solution (overnight) a small quantity of undissolved debris was removed by centrifuging in the Spinco model L preparative centrifuge in a No. 30 head for 30 minutes at 16,500 RPM.

The DNA in the supernatant was again precipitated with 1 volume of 95 per cent ethanol and resuspended in 200 ml citrate-saline. A second round of centrifugation in the Spinco was necessary, since there was persistent visible turbidity in the redissolved preparation. The DNA was finally precipitated with 1 volume of 95 per cent ethanol and resuspended in sterile citrate-saline. Analysis indicated a yield of 170 mg of DNA.

Assay Procedure For routine analysis of transforming activity of DNA, or for test of competence of cells, 0.1 ml of a DNA preparation appropriately diluted was added to 2.8 ml of Elev broth or 0.125 M saline. To this mixture 0.1 ml of 1 to 2 $\times$ 10^9 competent cells/ml was added and incubated with mild agitation for 30 minutes at 36°C $\pm$ 1°. Then 1 ml of suspension was introduced into a Petri dish or diluted, and 1 ml of the dilution added to the plate and mixed with 10 ml of Elev agar. The plates were allowed to harden for a few minutes and then incubated for 2 hours at 37°C, after which 10 ml of Elev agar containing twice the desired antibiotic concentration was added. After the agar hardened following this second addition, the plates were incubated at 37°C for a period of 18 to 40 hours.

The routine assay procedure used here was designed to measure the biological activity of transforming DNA or to evaluate the relative competence of different cell cultures. For most experiments, uptake of the DNA does not continue after 30 minutes. Consequently, it was not necessary to add DNAase to the mixture. Since the number of transformed cells did not increase appreciably until after 50 minutes at 35°C, there was ample time for dilution and plating at room temperature. For most assay procedures this technique was sufficient for determining transforming activity. For some experiments DNAase should be added to terminate the reaction (4).

Pour Plate Procedure Instead of incubating a transforming culture for 30 minutes,

incubation was extended to 2 hours and the culture then poured directly into liquid Elev agar containing the appropriate selective agent. In this case the concentration of antibiotic used was one-half the concentration used in the agar overlaying method. The concentrations of the antibiotics used in the experiments presented below are for streptomycin (Merck), a final concentration of 250μg/ml (base), or 500 μg/ml in the Elev agar overlay; cathomycin (Merck), 2.5 μg/ml; viomycin (Pfizer), 150 μg/ml; erythromycin (Lilly), 6.25 μg/ml, or 50 μg/ml for the Er^{50} mutant.

Preparation of Levinthal Stock for Growth of H. influenzae[1] To a 6 liter flask add 2100 ml distilled H_2O and 74 gm Difco dehydrated brain heart infusion. Bring this solution to a vigorous boil, remove from heat, and add cautiously 200 ml defibrinated sheep's blood. The defibrinated sheep's blood is obtained by stirring fresh blood with a rough wooden paddle immediately after it is taken from the animal. Stirring vigorously for 5 minutes with a motor-driven paddle or for 20 minutes by hand should be sufficient. The blood is strained through cheese-cloth and stored frozen before use. Medium made from unfrozen blood is usually turbid. The first addition of blood caused a heavy evolution of gas from the broth producing a violent foaming. Therefore, only 5 ml of blood should be added initially to the hot infusion, and no more added until the gas evolution ceases. Continue to add as rapidly as foaming will permit, until the entire 200 ml is introduced.

Swirl the flask of blood plus brain heart infusion a few times to mix the contents and allow the mixture to stand for a minimum of 1 to 2 minutes. Filter the mixture by gravity through Whatman No. 12 fluted filter paper into 4 liter filter flasks. The first portion of the filtrate coming through a fresh filter will be turbid and should be refiltered. (Be sure to fill the filter as full of liquid as it is going to be at any time during the subsequent filtering.) Collect all filtered medium into one container and dispense 53 and 210 ml (5 per cent allowance for evaporation) quantities into screw capped bottles.

Autoclave the bottles with lids loosened for 20 minutes after reaching a temperature of 118–120°C. Be sure the bottles are packed loosely and that the temperature of the autoclave stands at 99–100°C for 10 minutes before the escape valve is closed to raise the pressure. (In autoclaving large quantities of medium there is always danger that that the innermost bottles will not get up to temperature soon enough to be sterilized. On the other hand, excessive autoclaving of Levinthal medium destroys its ability to support growth of *H. influenzae*.) Do not tighten caps until bottles cool to room temperature.

The sterility of bottled medium was tested by incubating overnight with the lids loosened slightly. The lids were again tightened before moving the bottles so that the

[1] Since this work was completed, Mr. John Cameron and Mr. Harold Isaacson working in this laboratory have found that growth and development of competence of *H. influenzae* equal to that obtained with Elev broth were observed in a 3.5 per cent solution of Difco brain heart infusion mixed 3:1 with 3 per cent Eugonbroth appropriately supplemented with 10 μg/ml of hemin and 2 μg/ml of DPN. This medium has been used with uniform success for a number of months. It eliminates a time-consuming and apparently unnecessary step of the addition of fresh blood.

Mr. Marvin Talmadge of this laboratory has found that hemin as a 1 mg/ml solution in 4 per cent triethanolamine can be kept at 0°C for months if 1 mg *l*-histidine/ml is present and the solution is preheated at 60°C for 10 minutes.

liquid did not splash over the edge of the neck around the cap. Such splashing risks contamination of an otherwise sterile bottle. The bottles were then stored at 5°C until needed. To be sure that the medium was sterile, each bottle withdrawn from storage was incubated at 37°C overnight before using.

To prepare the final growth medium, Elev broth, the Levinthal stock solution was mixed 1:1 with sterile Eugonbroth (30 gm/liter H_2O) and 2 μg/ml sterile DPN was added. DPN stock solutions may be sterilized by filtration, not by heating.

TABLE I

EFFECT OF STAGE OF GROWTH ON THE FREQUENCY OF TRANSFORMATIONS

Cells from an overnight culture were diluted in Elev broth 1/15 in Experiment 1 and 1/20 in Experiment 2 (10^8/ml and 5×10^7/ml, respectively). At times indicated 0.1 ml was added to 2.9 ml of Elev broth containing 0.12 μg/ml of purified DNA obtained from streptomycin-resistant cells. The number of transformations and viable cells is expressed as the number per milliliter of culture before dilution into the transformation tube containing DNA.

Experiment	Stage of growth (turbidity)	Time	No. of transformations to streptomycin resistance	Viable cell count/ml
		min.		
1	0.22	80	3×10^5	
	0.26	125	7.5×10^5	
	0.30	160	7.2×10^5	
	0.33	195	3.3×10^6	
	0.40	270	1.5×10^6	
	0.46	325	3×10^5	
2	0.12	105	3×10^3	1.2×10^9
	0.23	160	9.6×10^5	2.4×10^9
	0.31	195	2.7×10^6	4.4×10^9
	0.40	255	4.5×10^6	7×10^9
	0.45	315	1.2×10^5	7.6×10^9

EXPERIMENTAL RESULTS

Interest in a better understanding of competence of *H. influenzae* stemmed from two objectives: first, to obtain consistently cells which gave a "high" frequency of transformations; and secondly, to determine whether there were any factors other than the cell and DNA which were required in this transformation process, particularly if there might be any agent produced by the cell which was involved in the transformation procedure.

New Method for Developing Competent Cells Alexander and Leidy (1) have shown that the *H. influenzae* transformed best when grown to the end of log phase or when approaching stationary phase of growth and this has been confirmed in this laboratory, as may be seen from the results shown in Table I.

Cells were diluted approximately 1/50 or 1/100 in Elev broth and shaken

with mild aeration in a rotary shaker. 0.1 ml aliquots of cells were removed at various times to determine competence, diluted with 2.8 ml of medium, 0.1 ml of DNA added, and assayed according to the procedure outlined previously. The results are outlined in Table I.

Since the highest frequency of transformation has certain theoretical as well as practical implications, those conditions were varied which might exist at the stage of growth of *H. influenzae* when they become competent. A state of partial anaerobiosis was imposed on the culture after a period of aerobic growth. This led to the finding that under these conditions, frequencies of

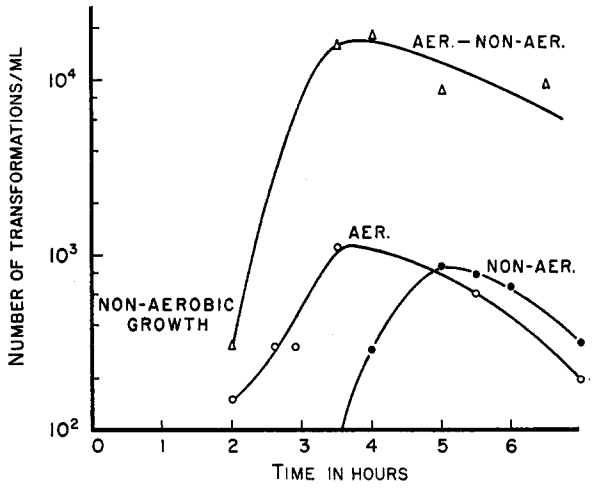

FIGURE 1. Effect of aerobic and non-aerobic growth on competence. From a previously grown culture (2×10^9/ml) 0.1 ml was inoculated into 3 ml of Elev broth in a 22 × 175 mm test tube with a 13 × 100 mm tube side-arm. Two cultures were shaken to produce "moderate" aeration and the other was incubated with the culture in the side-arm without shaking. At 2 hours one of the cultures was tipped into the side-arm while the other was allowed to grow aerobically. At the times indicated, samples were withdrawn and diluted 1 to 10 into Elev with 15 per cent glycerol and frozen. The transformation assay was done in the usual way.

transformation rose rapidly to 1 per cent or better for the streptomycin marker.

The procedure for preparing highly competent cells was as follows. Cells were diluted to a concentration of 2 to 4 × 10^7/ml and grown until they reached a concentration of 1 to 1.2 × 10^9/ml under conditions of moderate aeration. Aeration was obtained by using an Erlenmeyer flask set on a rotary shaker. The cells were then placed in a vessel with a restricted opening and held without agitation for 90 minutes. Fig. 1 shows the frequency of transformation as a function of this method for developing competent cells. 5 × 10^7 of these cells in saline containing 0.01 μg/ml or less of DNA removed approxi-

mately 30 to 50 per cent of DNA from solution. As the cell concentration increased, the removal of DNA approached 100 per cent. Competent cells prepared as noted above have been kept for periods up to 3 years with little or no change in competence by addition of glycerol to 15 per cent and storage at −60 to −70°C. Fox and Hotchkiss (5) have similarly found that glycerin aids in the preservation of cells at low temperature.

Evidence That Property of Competence Was Not Transferable Competent cell populations which were centrifuged and resuspended in fresh medium did

TABLE II

EFFECT OF RESUSPENDING MEDIUM ON COMPETENCE

Cells were grown aerobically to a concentration of 1×10^9/ml and divided into four portions. One was frozen in 15 per cent glycerol and designated as *Low competence cells*. The second was centrifuged to remove cells and obtain the *Supernatant of low competence cells*. The third and fourth cultures were incubated 90 minutes non-aerobically to a concentration of 1.7×10^9, one of which was designated as *High competence cells*, and the last centrifuged to obtain the *Supernatant of high competence cells*. The supernatants were heated at 60°C for 10 minutes to kill residual cells (the results are similar without heating). Samples of low competence cells (thawed) and of high competence cells were centrifuged and resuspended in the supernatants of high and low competence cells. The usual transformation assay procedure (see Materials and Methods section) was followed. DNA = 0.12 μg/ml (marker streptomycin resistance). Concentration of cells in assay tube = 5×10^7/ml. Transformations refer to number per milliliter in the assay tube.

Cells	No. of transformations in original medium	No. of transformations in supernatants of high competence cells	No. of transformations in supernatants of low competence cells
Low competence	10^1	10^1	2×10^1
High competence	6.6×10^5	6.4×10^5	7.5×10^5

not differ from uncentrifuged competent cells in their ability to be transformed. Therefore, one must conclude that the medium did not contain any factors due to cell metabolic activity which were required for transformation. When the cells were centrifuged and resuspended in saline, washed, and then resuspended again in saline, the cells lost 80 to 98 per cent of their capacity to undergo transformation (Table II). This suggested that there might be a factor in or on the cell which was important for transformation and that was removed by washing. Consequently, it was necessary to determine whether or not this material could be removed from one cell and transferred to another cell to make it competent. This notion was tested by centrifuging cell populations which were at a high or low level of competence and resuspending these cells in the supernatant of the opposite kinds. Under these conditions it was found that the cells retain the competence of the original cell population

(Table II). It was concluded from this that there was no transfer of a "competence" material in the supernatant.

The possibility remained that something is on the cell or is produced by the cell during the transformation process. If a transferable component responsible for the level of competence of a population were present during the transformation process, it would be expected that the competence of a population might be influenced by the presence of the cells which transform at a different frequency. Consequently, populations of low and high transforming cells were measured for their ability to transform in the presence of other high or low

TABLE III

FAILURE TO TRANSFER COMPETENCE

Rd cells were grown by the aerobic–non-aerobic technic. Er cells (low competence) were grown aerobically, the Er cells (high competence) were given an additional 90 minutes' growth non-aerobically. The cells were diluted to 5×10^7/ml in Elev broth to which was added DNA (streptomycin resistance) at a concentration of 0.12 μg/ml. Tubes 4 and 5 contained 5×10^7 cells/ml each of Rd and Er (low competence) or Er (high competence) cells. The streptomycin overlay was 500 μg/ml while the erythromycin was 12.5 μg/ml.

Tube No.	Cell marker	No. of transformations to: Streptomycin, Observed	Streptomycin, Expected	Streptomycin plus erythromycin
1	Er (low competence)	8×10^1	—	8×10^1
2	Er (high competence)	7×10^4	—	7×10^4
3	Rd	4.1×10^5	—	—
4	Rd and Er (low)	3.9×10^5	$4.1 \times 10^5(1+3)$	$1 \times 10^2(1)$
5	Rd and Er (high)	5×10^5	$4.8 \times 10^5(2+3)$	$7.6 \times 10^4(2)$

transforming cells. In order to identify one of the populations a strain resistant to erythromycin was chosen and its competence adjusted to the purposes of the experiment. Under these conditions cells of different competence, in mixed populations, retained the same capacity to undergo transformation as the original populations. The results are given in Table III.

It appears from the results in Table III that there was no change in competence on mixing cells of different competence so that competence is a property intrinsic to the cell itself or to a site on the cell. As will be shown below, however, it is capable of being influenced by additional external factors.

Competence Not Due to Genetic Factor Since the cells were competent as a result of growth to a fairly large population size, it was of interest to know whether or not there was a particular fraction of cells which was genetically distinct from the population as a whole. In a series of four tests in which forty colonies were isolated from viable count plates and made competent by the

method of Alexander and Leidy (1), there was no evidence that any of these colonies produced higher levels of transformation than others. This does not mean that competence is not genetically controlled, but places a restriction on the probable frequency of cells with different potential competence in a given culture. Determining the competence of cells which had already transformed would perhaps offer a more sensitive method for selection of highly competent cells.

The hypothesis was tested by two methods. First, cells that had undergone one round of transformation were selected and handled as in developing com-

TABLE IV

COMPETENCE DEVELOPED IN PREVIOUSLY TRANSFORMED CELLS

Rd cells made competent by the method of Alexander and Leidy by growth to turbidity of 0.38 were transformed with various crude DNAs. The DNA concentration was 0.1 μg/ml. Cell concentration was 2×10^8/ml.*

Cells	Transforming DNA	Frequency of transformation
Rd	Streptomycin resistance	4×10^{-4}
Rd	Viomycin	7.5×10^{-4}
Rd	Cathomycin	7.5×10^{-4}
Rd	Erythromycin	4×10^{-4}
Rd (S) (V)*	Cathomycin	3×10^{-4}
		4.2×10^{-4}
Rd (S) (V)*	Erythromycin	2×10^{-4}
		1.7×10^{-4}

* Rd (S)(V) cells made resistant to streptomycin and viomycin by transformation of Rd with DNA-Sr and DNA-viomycin were inoculated into Elev broth and grown to a turbidity of 0.38 and transformed with the DNAs indicated.

petence a second time. These showed no appreciable difference in frequency of transformation compared to cells which had not undergone a prior transformation. These results are given in Table IV. The second method subjected a competent population to one round of transformation and then grew this same population up to a competence state again and subjected the population to a second round of transformation. Under these conditions, the second round of transformation involved use of another genetically marked DNA to distinguish those cells which had or had not undergone transformation during the first treatment with DNA. Under these conditions it was observed that transformed cells showed the same capacities to transform as untransformed cells (Table V). Therefore, it is concluded that transformations were not restricted to a genetically distinct portion of the transforming population.

Environmental Conditions and Transformation The potentiality of the competent cell to react with DNA is limited by a number of environmental

factors. A few of the factors such as media, pH, salt concentrations, and temperature, which must be known if optimal conditions for transformation are to be obtained, will be considered.

Transformations at first were carried out in complex Elev medium but it was found subsequently that transformations would proceed equally well, if not better, in simple saline solution. Indeed, at low concentrations of DNA,

TABLE V

SIMILAR POTENTIAL COMPETENCE OF TRANSFORMED AND UNTRANSFORMED CELLS

Rd cells made competent by the aerobic-anaerobic method were transformed with DNA marked with streptomycin resistance and after removing an aliquot for the transformation assay, the culture was diluted $\frac{1}{10}$ into Elev broth and made competent a second time by the same method. These cells (Rd +Rd Sr) were then transformed with cathomycin and erythromycin transforming factors. DNA streptomycin = 0.12 μg/ml. 1st cycle cell concentration = 7×10^7/ml. DNA cathomycin = 0.16 μg/ml. 2nd cycle cell concentration = 8×10^7/ml. DNA erythromycin = 0.12 μg/ml.

First cycle

Cells	Transforming factor	No. of transformations S
Rd	Streptomycin	4.3×10^5

Second cycle

Cells	Transforming factor	No. of streptomycin-resistant cells	Viable cells	No. of transformations		Ratio	
				C	SC	C/Rd	SC/S
Rd + Rd Sr	Cathomycin	9.5×10^4	8×10^7	8.4×10^5	1.1×10^3	1.1×10^{-2}	1.2×10^{-2}
				E	ES	E/Rd	ES/S
Rd + Rd Sr	Erythromycin	9.5×10^4	8×10^7	4.7×10^5	5×10^2	5.9×10^{-3}	5.3×10^{-3}

transformations in saline may proceed at rates considerably above those in Elev broth. A typical experiment is shown in Table VI in which the rate in saline was three times the rate in Elev broth. The optimal concentration of saline was found to be 0.125 M. Some caution must be exercised with transformation in saline since *H. influenzae* loses its stability on prolonged exposure without the presence of protein or some stabilizing agent, a one to twenty dilution of Eugonbroth being satisfactory.

The kinds and concentration of ions in solution play an important role in determining the levels of transformation. When the transformations are carried out in a complex medium such as Elev broth, these effects, when low concentrations of ions are used, are not always consistent; *e.g.*, concentrations of Ca^{++} and Mg^{++} of 5×10^{-3} M and 5×10^{-4} M in a number of experiments

TABLE VI

KINETICS OF TRANSFORMATION IN BROTH AND SALINE

Competent Rd cells were diluted into two tubes, one containing Elev and one containing saline, in the following proportions: 0.1 ml Rd and 2.8 ml Elev or saline + 0.1 ml DNA (0.036 μg/ml), and incubated at 36°C for the times indicated. Aliquots were then diluted into broth containing 1 μg/ml DNAase and 0.005 M Mg^{++} and plated at appropriate dilutions, incubated 2 hours at 37°C, layered with streptomycin agar, and incubated as in the usual assay procedure. Final concentration of DNA = 1.2×10^{-3} μg/ml. Cells at start = 7×10^7/ml. At 60 minutes viable count in Elev = 2.7×10^8 and in saline = 1.3×10^8.

Time	No. of transformations to streptomycin resistance	
	Elev	Saline
min.		
2	3.3×10^2	1.5×10^3
4	8.5×10^2	4.0×10^3
8	3.0×10^3	8.8×10^3
16	1.1×10^4	3.0×10^4
20	1.3×10^4	4.0×10^4
30	2.3×10^4	7.7×10^4
40	3.0×10^4	9.8×10^4

TABLE VII

THE EFFECT OF ADDED MATERIALS ON TRANSFORMATION IN ELEV OR SALINE

The usual transformation assay was performed in the presence of added compounds with Elev broth or saline as diluent. DNA = 1.2×10^{-5} μg/ml; cells, 5×10^7/ml; pH of Elev, 7.4; pH of saline, 7.2

Experiment	Material added	Concentration in reaction mixture	Medium	Per cent of transformations in Elev without additions
1	PO_4, pH 7.4	6.7×10^{-2} M	Elev	6.2
		6.7×10^{-4}	"	100
2	NaCl	0.275 M	Elev	57
		0.2	"	78
		0.16	"	100
3	Glycerol	10 per cent	Elev	50
		1	"	100
		0.1	"	100
4	Citrate	10^{-2} M	Elev	69
		10^{-3}	"	90
		None	"	100
5	Citrate	10^{-2} M	Saline	46
		10^{-3}	"	108
6	Versene	10^{-3} M	Elev	100
		10^{-2}	"	0

proved to be stimulatory, but not always. However, in general, high concentrations of added ions and chelating agents tested proved to be inhibitory. The results of some of these tests are given in Table VII.

Capacity to undergo transformation was also affected by the hydrogen ion concentration of the solution. The process exhibited a pH optimum in saline as well as in Elev. Interestingly enough, these pH optima differed and the results for a typical experiment are given in Fig. 2.

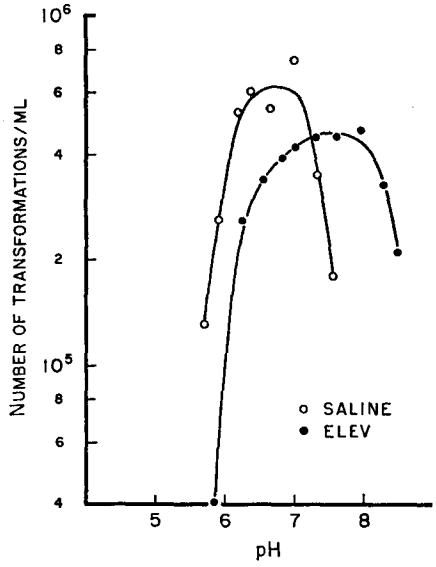

FIGURE 2. Effect of pH on transformation in growth medium and saline. The usual assay procedure was used except that the diluent was Elev broth or saline with 3×10^{-3} M PO_4 adjusted to the pH desired with N/10 NaOH or HCl. DNA at 0.12 μg/ml was added and then cells to final concentration of 5×10^7/ml. The pH was measured before and after the incubation period.

The transformation process also showed a temperature optimum as seen in Table VIII. These results are qualitatively similar to those obtained by others (5–7).

Loss of Competence by Washing and Its Restoration Although the state of competence (irreversible uptake of DNA) appears to be an intrinsic property of the cell, at least a portion of this mechanism may be removed from the cell and restored by appropriate treatment. Washing cells with saline removes a part of the ability of the cells to take up DNA, but to a far greater extent it affects the ability to complete the transformation process. The capacity to complete transformation can be restored by a number of treatments. The results of a typical experiment are given in Table IX. The results in Table X

show that cells, which on washing twice with saline had lost 97 per cent of their capacity to complete transformation, still took up more than 70 per cent as much DNA in terms of biological activity as the unwashed cells, but approximately 40 per cent of this DNA was removed by a single 2 minute washing; whereas, less than 1 per cent of the DNA could be removed from the unwashed cells.

Since no additional growth was detected in the restoration of competence to these cells, it seems likely that the restoration was the result of something which was supplied by the various factors and which was important either as

TABLE VIII

EFFECT OF TEMPERATURE ON THE NUMBER OF TRANSFORMATIONS

The usual assay procedure was followed except that the incubation of 30 minutes was performed in thermos flasks maintained at the temperature indicated. Cells made competent by the method of Alexander and Leidy; cell concentration 1.6 × 10^8/ml, DNA = 0.12 μg/ml (streptomycin resistance).

Temperature	No. of transformations × 10^{-3}
°C.	
10	20
25	187
30	567
34	878
37	612
40	125
43	45

cofactor or for synthesis to produce something essential for the irreversible step in the uptake of DNA and subsequent transformation.

Proportion of Competent Cells in a Population Only a portion of a population of cells undergoes transformation for a particular marker. Some populations of *H. influenzae* have yielded transformation frequencies of 10 per cent for the cathomycin marker and 5 per cent for streptomycin. In addition, frequencies of transformation for two unlinked erythromycin markers, viomycin and kanomycin, were approximately 8 to 10 per cent each. In any given population of cells the sum of the frequencies of transformation to individual markers suggested that under optimal conditions with only a limited number of markers at least 40 per cent of the cell population was competent. If the competence of a cell population is defined in terms of the ability to take up DNA, and subsequently undergo transformation, one would anticipate that the transformation of a population would be a function of the number of cells in the population which are capable of taking up DNA, the number of molecules taken up by the cell, as well as the number of kinds of DNA molecules in solu-

tion. The evidence for the presence of a mixture of molecules from transforming DNA will be presented elsewhere. For the moment, let us assume that the DNA preparation consists of a population of different kinds of molecules (the

TABLE IX

REDUCTION OF COMPETENCE BY WASHING AND ITS RESTORATION BY VARIOUS AGENTS

The usual assay procedure was performed except that 0.125 M saline was used as diluent and the cells were centrifuged, resuspended in saline, and then recentrifuged and resuspended either once (once washed cells) or twice (twice washed cells). The additions to the transformation tubes were made prior to adding the cells. The time for interaction between cells and DNA was 30 minutes, after which the usual dilutions in broth and plating procedure were carried out. The concentration of Rd competent cells = 5×10^7/ml final. The viable count at the end of 30 minutes indicated no loss of viability of the cells. DNA concentration; Experiment 1, 1.2×10^{-3} μg/ml, Experiments 2 and 4, 1.2×10^{-5}, Experiment 3, 0.12 μg/ml.

Experiment	Cells	Restoring agent	No. of transformations	Per cent of original competence
1	Washed once		2.7×10^4	11
			2.3×10^4	13
	Washed twice		9.4×10^3	4.5
			1.1×10^4	5.2
	Unwashed		2.1×10^5	100
2	Washed once	3 per cent Elev	2.7×10^3	25
	Washed once		6.9×10^3	63
	Washed once	Spermine, 3×10^{-3} M	6.1×10^3	55
	Washed twice		3.5×10^1	3.2
	Washed twice	3 per cent Elev	3.9×10^3	35
	Washed twice	Spermine, 3×10^{-3} M	2.9×10^3	26
	Unwashed		1.1×10^4	100
3	Washed twice		2.8×10^4	3.7
	Unwashed		7.5×10^5	100
	Washed twice	3 per cent supernatant	3.8×10^5	51
	Washed twice	6 per cent supernatant	5.6×10^5	75
	Washed twice	9 per cent supernatant	8.0×10^5	107
	Washed twice	12 per cent supernatant	8.8×10^5	117
4	Washed twice		1.6×10^1	2.5
	Washed twice	0.04 per cent bovine serum albumin	8×10^1	12
	Washed twice	3 per cent supernatant	3.7×10^3	58
	Unwashed		6.4×10^3	100

absolute number of molecules is not important as long as we assume a value of 10 or more). Then it is possible to estimate the number of competent cells in a population by an analysis of the number of transformations for two factors, these being a function of the number of transformations for single

factors. For example, in some experiments it was observed that frequencies of transformation to streptomycin or erythromycin were of the order of 1 in 1,000 to 1 in 5,000. However, the frequency of double transformation to streptomycin and erythromycin together was higher than expected from the product of the frequencies of single transformations. According to theory the probability of a double event occurring is simply the product of the possibilities of the two single events if the processes are independent. This discrepancy, which might have been due to linkage between the two markers, was also obtained when the factors were derived from two different cell populations re-

TABLE X

EFFECT OF WASHING ON THE COMPETENCE AND DNA BINDING OF CELLS

Rd cells made competent by the aerobic-anaerobic technique were washed twice with saline, 0.12 M, and resuspended in saline. The cells were diluted to a concentration of 5×10^7/ml in 0.125 M saline which contained 0.0012 μg/ml DNA. After 30 minutes' incubation the cells were centrifuged and resuspended in saline (0.15 M) and centrifuged again. The supernatants from each centrifugation were diluted and assayed against standard dilutions of the DNA used in the original assay.

Cells	No. of transformations	Per cent competence	Per cent of original transforming activity removed from solution	Per cent of original transforming activity washed from cells
Washed twice in saline	4.4×10^3	1.9	46.0	16.7
Washed twice in saline	7.0×10^3	3	70.0	30.0
Unwashed	2.3×10^5	100	94.2	0.6

sistant to the individual antibiotics; the markers could, therefore, not be linked. Therefore, if linkage was produced, it had to be the result of an interaction outside of the cell prior to infection of the host, and since no such interaction occurred for linked markers when they were added as two separate DNAs, this possibility was discarded. The higher frequency of double transformations than expected could be explained by assuming that only a fraction of the cell population is competent. This would raise the actual frequency of transformations above that computed from the viable count, especially of the doubles since the latter is the product of the individual or single frequencies. Perhaps this is more easily seen in the form of an equation: $(N_1/fB)(N_2/fB) = N_D/fB$ where N_1, N_2, and N_D are the number of bacteria transformed to characters 1, 2, and both respectively. B is the number of bacteria, and f the fraction that are competent. This then simplifies to: $f = N_1N_2/N_DB$.

In Table XI the fraction f of competent cells in two different populations was calculated. In Table XII the fraction of competent cells was found to be 0.68, and then the observed and calculated frequencies of double, triple, and

even quadruple transformed cells were compared. The transformations involving S and C markers required a correction for the recombinants involving the corresponding sensitive markers (12). The satisfactory agreement between calculated and observed frequencies in Tables XI and XII supports the assumption that the interaction of DNA and cells is random and also that the competence of a culture can be evaluated by the above method.

TABLE XI

CALCULATION OF COMPETENCY AS FUNCTION OF THE NUMBER OF DOUBLE TRANSFORMATIONS

High competence cells were grown by the aerobic–non-aerobic technique. The low competence cells were grown aerobically only. The concentration of DNA (marked with streptomycin and erythromycin) (SE) was 2 μg/ml and was a crude lysate. The results were exactly the same with purified DNA. The final concentration of antibiotics: streptomycin = 250 μg/ml; erythromycin = 25 μg/ml.

	High competency cells	Low competency cells
No. of viable cells	2.4×10^8	1.2×10^8
No. of transformations (observed):		
S	1.25×10^6	4×10^4
E	1.8×10^6	4.7×10^4
SE (double)	1.25×10^4	6.7×10^2
No. of SE expected:		
Random interaction $= \frac{S \times E}{\text{No. of viable cells}}$	9.4×10^3	1.9×10^1
No. of competent cells to give observed number of SE		
Doubles $= \frac{S \times E}{SE}$	1.8×10^8	2.8×10^6
Per cent competency $= \frac{\text{No. of competent cells}}{\text{No. of viable cells}} \times 100$	75 per cent	2.3 per cent

S, streptomycin-resistant.
E, erythromycin-resistant.
SE, doubly transformed to streptomycin and erythromycin resistance.

The number of cells used in the above calculations was determined by a viable or plate count, which unfortunately, is not identical with the total number of cells. Microscopic count indicated an average of 1.6 to 2 cells per viable cell unit. Extrapolation of the linear portion of the ultraviolet inactivation curve to the "0" dose for a population of cells gave a value of 1.5 to 1.6 cells per viable center. However, what was more important for the experiments reported above was that the inactivation of competent cells by ultraviolet radiation followed a single hit process, suggesting that the multicellular units did not appear to be multicompetent. The results of a typical experiment

in which the viable count and the number of competent units were inactivated as a function of ultraviolet dose are given in Fig. 3. The results were also the same for inactivation after reaction of the cells with DNA. One would expect, therefore, that in some highly competent populations the frequency of doubles might actually fall slightly short of the number expected randomly on the basis of the viable count. Such cases are found.

TABLE XII

FREQUENCY OF TRANSFORMATIONS TO ONE, TWO, THREE, AND FOUR FACTORS

The transformation was performed in the usual way except that the total volume, concentration of cells, and concentration of transforming DNA were increased. Rd cells were diluted to a concentration of 2.4×10^8/ml from 2.4×10^9/ml into 0.125 M saline with 10^{-3} M Mg^{++} plus 2 μg/ml each of crude DNAs unlinked markers C, V, and ES. After 30 minutes' incubation at 35°C the cells were diluted or not as required and plated in Elev agar and then after 2 hours' incubation at 35°C overlaid with the combination of antibiotics indicated. The final concentrations of antibiotics were: erythromycin, 25 μg/ml; cathomycin, 2.5 μg/ml; viomycin, 150 μg/ml; and streptomycin, 250 μg/ml. The frequencies observed and expected were based on 1.9×10^8 competent units/ml. The corrections for linked S and C were for 20 per cent linkage of S sensitive to C and 35 per cent linkage of C sensitive to S as previously determined for these DNAs.

	Observed	Expected	Expected corrected for linked S and C
Frequency of singles			
S	0.625×10^{-2}		
E	0.90		
V	1.9		
C	2.8		
Frequency of doubles			
SE	0.63×10^{-4}	0.56×10^{-4}	
SV	1.3	1.2	
SC	0.93	1.8	0.9×10^{-4}
EV	2.1	1.7	
EC	2.1	2.5	
VC	5.0	5.3	
Frequency of triples			
SEV	1.3×10^{-6}	1.2×10^{-6}	
SEC	1.2	1.6	0.84×10^{-6}
EVC	6.8	5.9	
VCS	2.4	3.4	2.6×10^{-6}
Frequency of quadruples			
SECV	2.0×10^{-8}	3.0×10^{-8}	2.1×10^{-8}

Cells: Rd.
Transforming factors: SE + CV.
S, streptomycin-resistant.
E, erythromycin-resistant.
C, cathomycin (novobiocin)-resistant.
V, viomycin-resistant.

The Limit of DNA Uptake by Competent Cells From the observations noted earlier by others (4, 13) and confirmed in this laboratory, that there is a limiting level of transformation as a function of increasing DNA concentration and that non-marker DNA interferes with transformation as a function of concentration of DNAs, it is concluded that there is a limit to the number of molecules

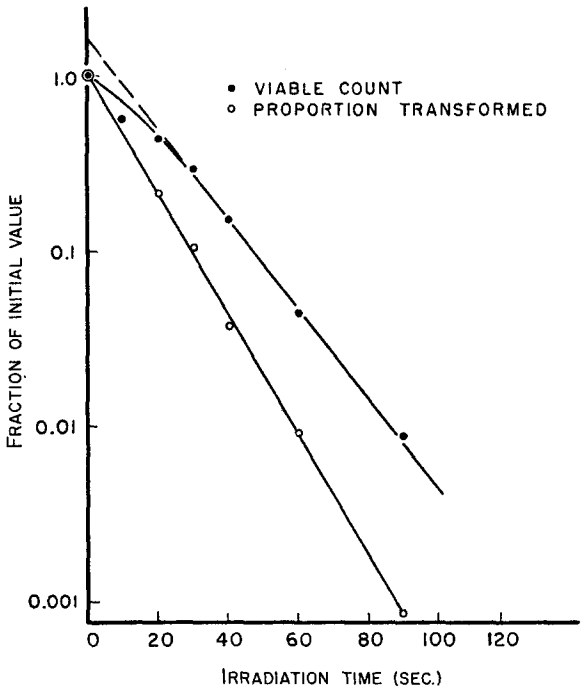

FIGURE 3. A comparison of the effects of ultraviolet light on cell viability and transformation. Two ml of cells made competent by the aerobic–non-aerobic method were mixed with 2 ml of Elev broth and irradiated with ultraviolet light in an open 100 mm Petri dish at 70 cm from a 15 watt germicidal lamp with shaking on an A. H. Thomas rotary shaker. At the times indicated, two 0.1 ml aliquots were removed. One was diluted into 1.3 ml of Elev. Later 0.1 ml of DNA (streptomycin-resistant) was added (concentration = 0.12 μg/ml). After 20 minutes' incubation DNAase was added to bring the concentration to 5 μg/ml. This was followed by the usual plating procedure. The second aliquot was diluted and plated for viable count.

which can be incorporated by a cell. The results in Tables XI and XII show that up to four independent nucleic acid units can be taken up by a single cell. Since the number of multiple transformations agrees reasonably well with that calculated on the assumption of random interaction of DNA and cells, it follows that virtually all the competent cells must be capable of taking up at least four molecules of DNA. The evidence from P^{32} uptake studies showed that *on the average* cells could take up three molecules per cell. These studies were

TABLE XIII

LOSS OF COMPETENCE BY GROWTH

Rd cells grown by aerobic growth to turbidity of 0.33 on the Coleman Jr. spectrophotometer at 650 mμ, which is equal to approximately 5×10^9 cells/ml, were diluted 1/30 into Elev. At 0, 15, 30, and 60 minutes 0.12 μg/ml of DNA with streptomycin resistance marker was added. 15 minutes after the addition of DNA, 1 μg/ml of DNAase was added. The tubes were then incubated for 2 hours at 37°C, diluted, and plated with 500 μg/ml streptomycin agar. The colony counts were made after 18 hours' incubation.

Time of incubation before DNA added	No. of transformations in transformation tube
min.	
0	1.2×10^5
15	1.6×10^5
30	3×10^4
60	$< 10^1$

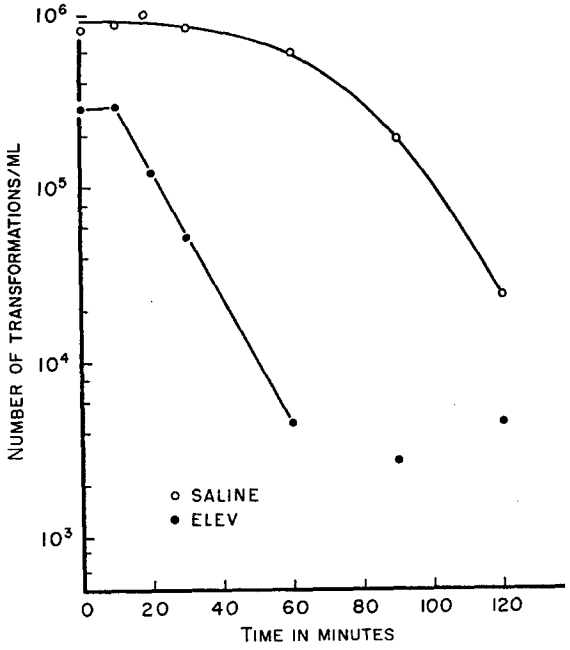

FIGURE 4. Loss of competence in growth medium and saline. Competent cells were diluted 1/30 (6×10^7/ml final concentration) into two series of tubes containing either Elev broth or saline. The tubes were incubated at 35°C and at the various times indicated, the competence of the cells was determined by the addition of transforming DNA (0.12 μg/ml streptomycin resistance) to the individual tubes. The incubation time after the addition of DNA was 30 minutes in all tubes. The number of transformations in the tubes is plotted as a function of the time of incubation in Elev broth or saline before the addition of DNA.

performed with cells with less competence than is now available and it has been found that the number of molecules removed per cell can be increased (10). However, there is no doubt of the uptake of three or four molecules per cell, which then makes it necessary to consider what factors limit the number of transformations in the presence of excess DNA. This question will be considered in the succeeding section.

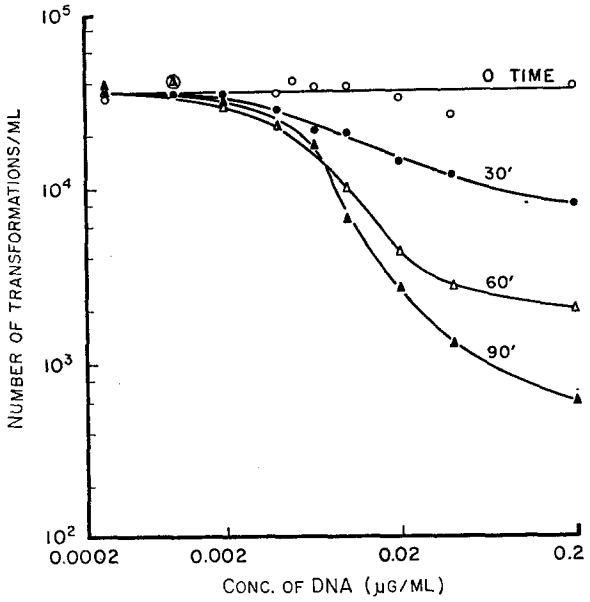

FIGURE 5. Effect of DNA concentration on loss of competence. Cells (6×10^7/ml) were incubated in saline with DNA (cathomycin resistance) at various concentrations for 0, 30, 60, and 90 minutes. Samples were diluted into saline containing a 1/30 dilution of broth plus 0.12 μg/ml of DNA (Sr). After 30 minutes at 35°C the samples were diluted, plated, and after 2 hours at 37°C, layered with 500 μg/ml of streptomycin. The number of transformations to streptomycin resistance is plotted against the concentration of DNA in the first incubation tube as follows: 0 minutes, open circles; 30 minutes, closed circles; 60 minutes, open triangles; 90 minutes, closed triangles.

Loss of Competence as a Result of Interaction between the Cell and DNA It has been observed that the ability of cells to maintain competence is transient. For pneumococcus at 37°C this period is of the order of 10 minutes (7, 8), for *Hemophilus* somewhere between 20 and 30 minutes. This loss in competence seems to be related to the metabolic activity of the cells (7). (Thomas (7) observed that the loss in competence was temperature-dependent.) Fox and Hotchkiss (5) found that the dilution of freshly thawed pneumococcal cells into the growth medium produced a linear increase in the number of transformants over a period of time greater than 1 hour, during which time the

cells were not dividing. When competent *Hemophilus* cells were diluted in saline containing a small amount of nutrient broth, the competence of this population was maintained for greater periods of time than for a similar dilution of cells into growth medium (Elev). The results of two experiments are given in Table XIII and Fig. 4. In the saline experiment some decline in competence was observed after 60 minutes' incubation, but a number of experiments have been performed in which the competence level was maintained without loss for 90 to 120 minutes. If the cells were incubated in dilute medium

TABLE XIV

LOSS OF COMPETENCE IN SALINE DUE TO DNA UPTAKE

Competent Rd *Hemophilus* cells, 1.2×10^8/ml in 0.125 M saline, were incubated with or without 0.2 μg/ml of DNA (marked with cathomycin resistance) for 0, 30, or 60 minutes at which times samples were diluted 1/10 into saline containing 1/15 broth and 0.5 μg/ml of a DNA preparation from cells resistant to both streptomycin and erythromycin. This mixture of cells and DNA was incubated for 30 minutes, diluted appropriately, and assayed for transformations to streptomycin and erythromycin resistance. The dilution mixture for cells incubated previously without DNA also contained 0.02 μg/ml of DNA (cathomycin resistance). Appropriate controls were also included.

Time of contact with initial DNA (cathomycin resistance marker)	No. of transformations/ml			
	Streptomycin resistance		Erythromycin resistance	
	Experimental	control*	Experimental	control*
min.				
0	2.4×10^4	1.8×10^4	3.5×10^4	3.3×10^4
30	6.9×10^3	1.5×10^4	1.4×10^4	3.1×10^4
60	2.0×10^3	1.6×10^4	4.3×10^3	2.8×10^4

* Saline was substituted for the DNA marked with cathomycin resistance during the initial exposure of the cells.

containing DNA, the cells gradually lost the capacity to react with other DNA. Indeed, the loss in capacity of the cells to take up DNA was a function of the concentration of DNA with which they had been previously incubated; these results are given in Fig. 5 and Table XIV. These results can be interpreted in two ways. First, the original incubation of DNA covered up the sites which were necessary for reaction with the second preparation of DNA. Second, exposure of the cells to DNA actually initiated the process by which the cells lost competence. These two possibilities were tested by exposing the cells to DNA, centrifuging to remove exogenous DNA, and then following the loss in competence in these cells. The results are given in Fig. 6. It is clear from these results that after a cell has reacted with DNA, its affinity for reacting with additional molecules of DNA is lost as a function of the time after the cells are exposed to the original DNA. Indeed, when the fraction of cells retaining their

competence was plotted against time after exposure to DNA, a straight line relationship was obtained which went through the origin indicating that a single interaction between DNA and the cells was sufficient to initiate the loss of competence.

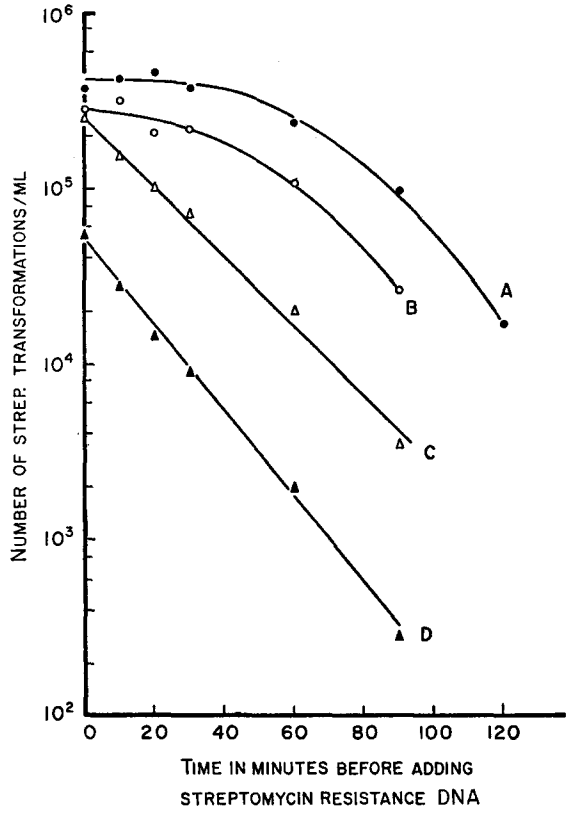

FIGURE 6. Effect of time after mixing with DNA on loss of competence. Rd cells made competent by aerobic–non-aerobic treatment were diluted 0.1 into 2.8 ml of saline (5×10^7/ml) and incubated with DNA carrying a cathomycin marker for 0, 5, 30, or 60 minutes (curves A, B, C, D respectively). At these times the cells were centrifuged and resuspended in 3 ml of saline with 1/30 broth and incubated at 35°C for the time shown on the abscissa. At this time 0.12 μg/ml of DNA with streptomycin marker was added and incubation continued for 30 minutes more to measure the level of competence remaining; *i.e.*, the number of transformations to streptomycin resistance as a function of time of incubation in saline. In the control the cells were incubated initially without DNA.

DISCUSSION

The model which is discussed for the reaction between DNA and the cell leading to transformation is by no means a new one (5, 7). However, our data show some points of disagreement as well as agreement with the models which have been presented previously.

Hotchkiss, Thomas, and others have found that the number of transformations in pneumococcus is limited by the length of time that the cell remains competent, which in their system is of the order of 10 minutes. Thomas, starting with a given competent population, measured the loss in competency of transformable cells and the effect of DNA concentration on transformation and derived a relationship which indicates that the limiting number of transformations obtained in excess concentrations of DNA is a function of the number of competent cells. Fox and Hotchkiss (5), using the treatment conventional in enzyme studies, have derived relationships consistent with the idea that there is a reversible reaction followed by an irreversible reaction between the cell and DNA, and when they measured the kinetics of transformation both as a function of cell concentration and DNA concentration, they estimated the number of attachment sites per cell to be from 33 to 70 per pneumococcal cell.

In the case of *H. influenzae* under normal growth conditions, competence is lost after 20 to 30 minutes' incubation, but if the cells are incubated in a restricted medium, the cells will remain competent for much longer periods of time unless the cells react with DNA. The present evidence suggests that once the cell has reacted with DNA it begins to lose competence according to first order kinetics; *i.e.*, DNA when taken up irreversibly itself stimulates the cell's metabolic reactions in favor of the loss of a material or condition necessary for the uptake of DNA. Some preliminary evidence suggests also that the step lost when cells are incubated with broth or with DNA is the reversible step (9).

In *H. influenzae* as in pneumococcus (5), at high DNA concentrations there is a maximum rate of irreversible uptake of DNA by the cell. It follows, therefore, that the maximum number of transformations is determined by the length of time the cell remains competent. In the case of transformation in broth, loss of competence is conditioned by the growth of the cell; in the case of transformation in saline it is conditioned by the loss of competence of cells which have reacted irreversibly with DNA. The following scheme may be diagrammed:

$$B \rightarrow B\cdot\text{comp.} + \text{DNA} \rightleftharpoons B\cdot\text{comp.}_{t_1} + \text{DNA} \rightleftharpoons$$

Broth 35°C ↑↓ B_i ; k_{i_1} ↓ B_{t_i}

$$B_{t_1}\cdot\text{DNA} \rightleftharpoons B\cdot\text{comp.}_{t_{1+n}} + \text{DNA} \rightleftharpoons \text{etc.}$$

k_{i_2} ↓ $B_{t_{i+n}}$

where B represents the cell which is not competent, B_i represents cells which are no longer competent, B_{t_1} represents a cell which has taken up a DNA molecule and is therefore a potentially transformed cell for one factor, $B_{t_{i+n}}$ represents a cell which has taken up more than one DNA molecule and is therefore a potential double transformation. k_{i_1} and k_{i_2} are the rate constants for the reactions taking competent cells to an incompetent state. In broth at 35°C the reaction $B \cdot \text{comp.} \rightarrow B_i$ is faster than the rate in saline; *i.e.*, the equilibrium is in favor of the loss of competence.

This scheme is formally analogous to Thomas's except that the assumption that the interaction between DNA and the cell does not affect competence has been dropped. However, in the case of pneumococcus transformations in broth, this effect may be a relatively small one.

The scheme which is proposed visualized the cells of a transforming population as having a number of receptive niches or sites with which different molecules of DNA may react. In excess DNA the DNA is taken up and limits the time during which the niches can remain open, which is of the order of 20 to 30 minutes in saline. During this time the probability of incorporating any marker is random and the probability of multiple uptake is merely the product of the probability of uptake of the single markers.

It is clear that before the initiation of transformation the cell must develop the capacity to take up DNA as well as to incorporate it into its structure. Cells which are growing logarithmically in Elev broth do not have the capacity to take up DNA reversibly (9). Whether or not they possess the mechanism for the incorporation of DNA after reversible uptake is, of course, not known. After the cells develop the capacity to take up DNA reversibly it is found that they also have the capacity to take up DNA irreversibly. Cells made competent by the method of Alexander and Leidy, lose the capacity for irreversible uptake if growth is renewed, but still maintain the capacity for taking up DNA reversibly. The irreversible uptake of DNA may be prevented by washing competent cells; however, a large part at least of the ability of the cells to take up DNA reversibly is still maintained, and in fact the ability of the cell to take up DNA irreversibly can be restored by incubation of the cells in a small amount of growth medium which is insufficient to permit division of the cells. The fact that the irreversible step can be modified without appreciably affecting the amount of DNA bound reversibly suggests that the two processes represent separate biochemical events. These effects seem to be similar to those described by Fox and Hotchkiss (5) for the restoration of transformability of frozen pneumococcal cells.

Of considerable interest is the finding that cells which are grown aerobically or non-aerobically do not develop a high level of competence, but cells which are grown in an aerobic state and maintained in a reduced aerobic condition become highly competent. However, restricted growth is certainly not suf-

ficient in itself to produce competence since reduced aerobic growth alone does not produce a high level of competence and in addition it is clear that the cell must produce some material or condition on its surface which is capable of permitting reversible uptake of DNA. The fact that competent cells which can bind almost all the DNA in solution in an irreversible form, can be made to lose the irreversible step and still bind DNA reversibly suggests that the reversible uptake of DNA is a prelude to the irreversible uptake (5).

From those experiments in which cells have gone through two cycles of competence as well as the fact that reisolation of single cell cultures over a period of several years did not improve the competency of cells, it was concluded that there was no genetically distinct proportion of the population which was competent. From those experiments in which transformations were performed in the presence of both competent and non-competent cells, it was demonstrated that there was no transfer of competence from one cell to the other. Indeed, cells which had lost their competence by incubation in media did not show any increase in the presence of highly competent cells. It is concluded, therefore, that competence cannot be transferred when either the irreversible step or both the irreversible and reversible steps in DNA uptake are absent.

Competence then is a property intrinsic to the cell itself which results from the development of a reversible mechanism for taking up DNA and an irreversible mechanism which probably involves at least two components, one of which may be removed by washing and restored by external factors, the other of which must develop during anaerobic growth. In addition, the interaction between DNA molecules and the cell is a random event.

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