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Emergence of Competence (for Transformation) of Three *Hemophilus* Species in a Chemically Defined Environment.† (27904)

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A number of factors determine the proportion of cells in a bacterial population in which a new genetic trait may be conferred by biologically active deoxyribonucleic acid (DNA)(1). Among these, the method of cultivation of recipient populations plays a significant role. Under our original method of propagation of *Hemophilus influenzae*, the highest frequency of transformation to streptomycin (SM) resistance was approximately 0.1%. Goodgal and Herriott(2) have shown that the proportion of competent (transformable) cells in R*d* *H. influenzae* populations may be increased to 1% or more if the population is aerated during exponential growth and then, after a period of incubation with reduced aeration, is diluted 20-fold into NaCl containing DNA and incubated for 30 minutes. Some unknown factor(s) in the culture medium appeared to be essential for the high degree of competence of the diluted population; in our experience dilution of even 1:100 in saline leads to a marked decrease in the transformability of the population. This paper reports the use of this "dilution effect" as a tool for development of a chemically defined medium in which a high degree of competence emerges even in 10^4-fold dilution in 3 species of *Hemophilus: H. influenzae, H. aegyptius* and *H. parainfluenzae*. The results suggest that 2 amino acids, L-aspartic and L-glutamic, play a significant role. The process required for emergence of competence in the defined medium is temperature dependent and is inhibited by chloramphenicol.

Materials and methods. Recipient populations: Strains R*b*, R*d* and S*d* *H. influenzae* (3), *H. parainfluenzae* # 7 (Boss)(3), and *H. aegyptius* # 15 (4). *DNA donor populations:* Spontaneously occurring mutants resistant to streptomycin were selected from members of the above 3 species for isolation of DNA. The method used has been reported(5). Homologous species DNA was used in final concentrations of 1 or 2 μg/ml. *Medium used for growth of recipient populations:* Elev. broth(2) was used for the growth of test populations. Levinthal agar was used for pour plate preparations. The Levinthal stock was sterilized by filtration through Seitz filters. *Synthetic medium used for expression of competence:* Components of the medium were: 1) potassium phosphate buffered saline, pH approximately 7.5 (final concentration 0.015 M with respect to the buffer, 0.15 M with respect to NaCl); 2) L-aspartic and L-glutamic acids* in concentrations of 0.5% to 1.0% (w/v). One percent aqueous solutions were adjusted to a pH in

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the range of 7.2 to 7.8 with 1 N NaOH. The use of sterile distilled water as solvent and heating of the solutions in a boiling water bath for 5 minutes eliminated the necessity of autoclaving solutions for sterilization; 3) calcium chloride and magnesium sulfate in concentrations of 10^{-4} M and 4×10^{-4} M respectively. *Preparation of competent populations:* Recipient populations were propagated by the method of Goodgal and Herriott (2). For the populations studied by us, 16 hour subcultures of recipient populations were seeded into small volumes of Elev. broth in 125 ml Erlenmeyer flasks. The inoculum size and volume of medium varied with the recipient populations used, since growth rates varied. Cultures were incubated at 35° to 37°C with aeration provided by a magnetic stirrer or a mechanical shaker (approximately 125 rpm); they were incubated until a density corresponding to 1.5×10^9 to 2×10^9 colony forming units per ml was reached (reading of 70 to 100 with Klett-Summerson colorimeter (# 54 filter)). Populations increased approximately 4 generations during this growth phase; 2¼ to 3¼ hours incubation, depending upon the strain used, was required for this increase. The culture was then incubated (reduced aeration) for 1½ hours (37°C) in the tube used to measure its density (standardized tube for Klett-Summerson colorimeter). Populations increased by approximately ½ to 1 generation during this period (reading of 100 to 140 with the densitometer). The competence of populations at the end of the reduced aeration growth phase was measured in most experiments reported. An aliquot was removed 10 or 15 minutes before the end of this phase and DNA added for a 10- or 15-minute exposure period. This procedure provided a measure of the competence of a population prior to its dilution 10^{-1} into saline or 10^{-4} or 10^{-5} into the defined medium and is referred to as the "zero time" control. Controls of 3 other types were included: the "standard" culture diluted 1:10 in phosphate buffered saline and maintained at 30°C for 30 to 60 minutes before exposure to DNA for 10 or 15 minutes(6) as the "10^{-1} saline" control, cells not exposed to the DNA and cells exposed to deoxyribonuclease inactivated DNA. The method reported earlier was used to examine for transformants(3).

Experimental results. Goodgal and Herriott(2) report that 1% or more of an *H. influenzae* population may be transformed with respect to a particular marker if, after a period of growth under aerated and nonaerated conditions, the population is diluted 20-fold into saline (or broth) containing the marker DNA and further incubated for 30 minutes; higher dilution in saline leads to a decrease in the competence of the population. Goodgal and Herriott(2) refer to the "aerated, nonaerated" culture as the "highly competent" culture, but our experience confirms the observation of Stuy(6) that the high degree of competence is reached only after dilution and incubation in the saline or broth. The "aerated, nonaerated" culture is hereafter referred to as the "standard" culture.

If the "standard" culture is diluted 1,000-fold or more, instead of 10-fold, in buffered saline and incubated for 30 or 60 minutes, the competence of the population is not enhanced. A decline in viability of the population may occur at dilutions greater than 1,000-fold. A measure of the competence after this degree of dilution in saline provided a tool for exploring chemically defined conditions under which an enhancement occurred that was comparable to that obtained by a 10-fold saline dilution of the "standard" culture.

The basic medium initially used in this study was the "simplified" synthetic medium reported by Herbst and Snell for growth of a strain of *H. parainfluenzae*(7). Our strains of *H. parainfluenzae* required the addition of histidine for growth. Competence of *H. influenzae* populations diluted 10^3-fold or greater and incubated in this medium was comparable to the "10^{-1} saline" control. This observation and study of the influence of individual amino acids on transformability led to the development of the defined medium, composed of buffered saline, calcium and magnesium salts and L-glutamic and/or L-aspartic acids *(Materials and methods)*. The effectiveness of these 2 amino acids (singly

TABLE I. Competence of "Standard" *Hemophilus* Populations Diluted and Incubated for 1 Hour in Defined Medium. Measured by transformation to streptomycin (SM) resistance.

Recipient populations				SM resistant transformants per ml of diluted population					
				Controls‡		After incubation in defined medium containing L-aspartic and/or L-glutamic acid in concentrations indicated			
Species	Strain	Dilution† of "standard" culture examined	Exp	Zero time	10^{-1} in saline	1% L-aspartic	.9% L-aspartic .1% L-glutamic	.5% L-aspartic .5% L-glutamic	1% L-glutamic
H. influenzae	R*d**	10^{-5}	1	17	350	355	300	230	265
			2	15	320	210		428	218
			3	5	190	78	255	243	233
	S*d**	10^{-5}	1	1	154	256	293	468	322
			2	6	50	120	425	380	298
	R*b**	10^{-4}	1	<1	305	530	570	1033	945
			2	3	320	228	385	410	385
H. aegyptius	15	10^{-4}	1	<1	177	641		600	662
			2	<1	304	208		286	265
H. parainfluenzae	Boss	10^{-4}	1	57	735	218	315	765	673
			2	87	993	338	520	860	928

* R = nonencapsulated; S = encapsulated; small letter = type of origin.

† 10^{-5} dilution = approximately 3.5×10^4 colony forming units/ml. 10^{-4} dilution = approximately 3.5×10^5 colony forming units/ml.

‡ Controls: After DNA exposure, diluted appropriately.
Zero time = approximately 2.5×10^9 colony forming units/ml undiluted. 10^{-1} in saline = approximately 3.0×10^9 colony forming units/ml undiluted.

Defined medium = K phosphate buffered saline + 10^{-4} M $CaCl_2$ + 4×10^{-4} M $MgSO_4$ + concentrations of amino acids indicated.

or in combination) in promoting competence in populations of 3 species of *Hemophilus* is shown in Table I. Two types of controls were used to measure degree of enhancement in the defined medium; one measured the competence of the "standard" population at the time it was diluted into the medium and is referred to as the "zero time" control *(Materials and methods)*; the other measured degree of enhancement reached after dilution of the "standard" culture 10-fold in buffered saline and incubation for 60 minutes before exposure to DNA for 15 minutes. The latter served as an index of the expected maximum transformability of the population and is referred to as the "10^{-1} saline" control. "Standard" populations of each recipient strain were diluted 10^4- or 10^5-fold into the defined medium containing the amino acids in the concentrations indicated and incubated at 30°C for 60 minutes prior to addition of DNA. Exposure to DNA was limited to 10 or 15 minutes; deoxyribonuclease (DNAase) was added to stop the reaction.

It is apparent that a 4-fold or greater increase in number of competent cells occurs in the medium when the amino acids are used singly or in the combinations listed, but that when used singly, L-glutamic acid is in general superior to L-aspartic for the *H. influenzae* and *H. parainfluenzae* populations. In the *H. aegyptius* population, the 2 amino acids showed similar effects. The data show that 90% or more of those cells which become competent are shown to attain the competent state after incubation in one or more of the variations of the chemically defined environment.

Competence in these experiments is measured by the number of transformants per ml rather than their frequency since there is an increase (generally the equivalent of ⅓ to ½ a generation) in viable cell centers after incubation for 1 hour in the defined medium. Since the medium does not support the growth of the populations used, this increase may reflect the separation of aggregated cells, completion of the division of partially divided cells or other unknown factors.

Since L-aspartic acid at times was more effective than L-glutamic acid in enhancing the competence of R*d* populations, and since the medium which contains the 2 amino acids was as effective as the better of the single amino acids, the use of both amino acids in the medium was adopted for further experiments with populations of all 3 species of *Hemophilus*. In a total of 28 experiments (strain R*d*) in which the medium contained both amino acids, only 2 have shown a degree of competence significantly lower than the "10^{-1} saline" control.

Our results suggest that incubation in the defined medium for at least 60 minutes is required for development of a degree of competence comparable to that of the "10^{-1} saline" control, as shown in Fig. 1, for strain R*d* *H. influenzae*. For Exp. I, a 10^4-fold dilution of the "standard" culture was incubated and aliquots removed after each 30 minutes and exposed to the DNA for 10 minutes; competence in the defined medium reached the control after 60 to 90 minutes incubation. For Exp. II, DNA was added at the beginning of incubation in the medium, and the number of transformants measured after the periods indicated. A decline in competence may occur with incubation longer than 90 minutes (Exp. I). The rate at which competent cells appear in the control and defined medium are similar in Exp. II.

Addition of either the calcium or magnesium salt to the buffered saline containing L-aspartic and L-glutamic acids enhances the competence which emerges in the *H. influenzae* populations. At the concentrations adopted for use (4×10^{-4} M $MgSO_4$ or $MgCl_2$ and 1×10^{-4} M $CaCl_2$), the stimulatory effect of both salts is greater than either one alone; at least 33% more transformants emerged in the medium which contained both salts. However, addition of the magnesium salt alone in concentrations of 10^{-2} M or 10^{-3} M was as stimulatory for the competence of *H. influenzae* (R*d*) as addition of both salts in the concentrations adopted for routine use. The calcium salt alone at 10^{-4} M was not stimulatory for the *H. aegyptius* population in 2 of 3 experiments; at a concentration of 10^{-3} M, the salt depressed the emergence of competent cells. This depressing effect was not noted in the *H. influenzae* popula-

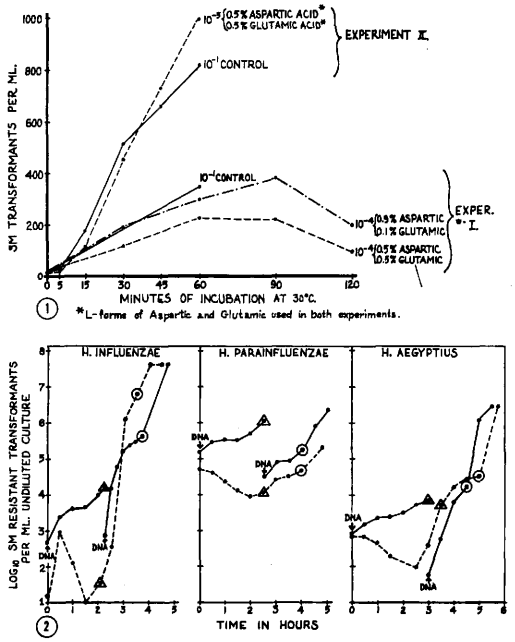

FIG. 1. Time required for R*d* *H. influenzae* in defined medium at 30°C to reach maximal competence for transformation to streptomycin resistence.

FIG. 2. Competence of 3 species of *Hemophilus* during aerated and nonaerated growth and after subsequent saline treatment.

•——• DNA added at commencement of aerated and nonaerated phases and at end of saline treatment. DNAase added at time intervals selected.

•– – –• Separate aliquots removed and exposed to DNA for 10 or 15 min, then DNAase for 3 min.

▲ End of aerated phase.

⊙ End of nonaerated phase.

Saline treatment—"standard" population diluted 10-fold in buffered saline.

tions; in some experiments the higher concentration (10^{-3} M) was more effective than the lower (10^{-4} M) in enhancing competence.

The bivalent cations are not stimulatory for competence of the *H. parainfluenzae* population but their presence does not appear to depress the emergence of competent cells. It is of interest that sodium citrate (0.02 M) prevented the emergence of competence when the bivalent cations were omitted from the medium.

Although one function of the L-glutamic and L-aspartic acids for the *H. influenzae* and *H. aegyptius* populations is to preserve the viability of cells, an unknown specific function is also suggested for at least the *H. influenzae* population. For example, a number of other amino acids in concentration of 1% were effective in preventing a loss in viability of the population but were virtually ineffective in promoting the appearance of competence: L-lysine, L-methionine, L-histidine, DL-valine, L-leucine, DL-isoleucine, L-phenylalanine, L-proline, L-glycine, and L-arginine. A loss in viability of the population occurred in the presence of 1% L-cysteine. Monosodium glutamate was highly effective in promoting the emergence of competence. Neither L-asparagine, L-glutamine, D-glutamic nor D-aspartic acids were effective substitutes for L-glutamic and L-aspartic acids.

Other substances were examined as substitutes for L-aspartic and L-glutamic acids; sodium salts of pyruvic, succinic, oxaloacetic, malic and α-ketoglutaric acids and sodium acetate. Incubation in their presence for 1 hour led (as did incubation in buffered saline) to a marked decline in the viability of the test population (strain R*d*). No decline in viability occurred in the presence of α-ketoglutaric acid in combination with leucine or lysine, but competence was not significantly enhanced. Glucose (1 mg/ml) was not stimulatory for competence when added to the medium with or without L-aspartic and L-glutamic acids.

The emergence of the competent state in the synthetic medium is temperature dependent. No significant difference in number of transformants was noted in R*d* (*H. influenzae*) populations incubated at 30°C and 37°C; there was a reduction (75% to 90% of the control) in the populations incubated at 22°C. No transformants were observed at 43°C even though there was no loss of viability of the population after incubation for 1 hour at this temperature.

Crystalline bovine albumin (1%) may or may not increase the rate at which competent cells arise in the synthetic medium, but is not necessary for their emergence. With strain R*d* as test population the action of albumin, when stimulatory, occurred in the first 45 minutes of incubation and resulted in a doubling of the number of transformants found in the control of the medium without albumin.

Chloramphenicol (1 μg/ml) inhibits the

emergence of competence of the "standard" populations when diluted and incubated in the defined medium. After 15 or 20 minutes incubation in the presence of the drug, no significant increase in number of transformants occurred in populations of the 3 species examined. Since there was no decline in viability of the populations within the 1 hour incubation period, and treated populations were diluted to subinhibitory concentrations of the drug, the data suggest that protein synthesis is necessary for the emergence of competence in the synthetic medium.

Competent populations may be washed at least twice in the synthetic medium without that loss of competence which occurs on washing in buffered saline. The competent population (R*d*) was prepared by diluting a "standard" culture 10-fold in buffered saline and incubating the diluted population for 30 minutes at 30°C. Aliquots of this competent population were centrifuged at 2,000 rpm for 25 or 30 minutes at 20°C; the sedimented cells were resuspended to volume in buffered saline or the synthetic medium. The competence of populations after the first wash was measured immediately and also after it had been diluted 10^4-fold and incubated for 1 hour in the defined medium. Exposure to DNA was limited to 10 minutes. Cells which were washed twice were immediately exposed to the DNA. The data show not only that a competent population may be washed at least twice in the defined medium and retain its competence, but also that the competent state may be "restored" in the population after it is lost by a wash in buffered saline.

The demonstration that competence can emerge or be "restored" in the synthetic medium raised the question as to whether the competence which evolves in the "10^{-1} saline" control or the synthetic medium is a reflection of the "restoration" of competence to cells which became competent at an earlier period during growth but subsequently lost their transformability. The competence of populations during both the aerated and nonaerated periods of growth was therefore measured. Two methods were used: 1) DNA was added at the beginning of each of these two phases and 2) aliquots of each phase were removed at specific intervals of incubation and the cells exposed to the DNA for 10 minutes. At the end of the nonaeration phase, the populations were also diluted 10-fold into buffered saline, incubated at 30°C for 30 and/or 60 minutes, and exposed to the DNA for 10 minutes; this procedure provided a control of the maximum expected transformability of the population. Populations of 3 species of *Hemophilus* were examined; the results are shown in Fig. 2. A measurement of competence at varied intervals in the absence of DNA shows a drop in transformability during the aeration phase and rise during the nonaeration phase.

It is apparent that during growth of populations of all 3 species competence at no time reaches the level attained after a 10-fold dilution and incubation in saline. This point is emphasized in those experiments in which the DNA was added at the beginning of each phase of growth. There is a slight increase at the beginning of the aerated phase, but no further increase during this phase until that period is reached when cells competent within the first 30 minutes should have begun to multiply as SM-resistant cells. The increase during the nonaeration phase is real since 1½ hours incubation is expected to be sufficient for the expression and possible replication only of those cells which were initially competent.

The data of Fig. 2 are interpreted as evidence that, for the *H. inflenzae* and *H. aegyptius* populations at least, virtually all cells which become competent do so during the period of exposure to the saline-diluted broth environment. This observation confirms that of Stuy(6) with respect to *H. influenzae*. It is apparent also that a measure of transformability at the end of the nonaeration phase is a measure of the highest degree of competence attained up to that point in the growth of the recipient population. Therefore, the high degree of competence which has been shown to emerge in the *H. influenzae* and *H. aegyptius* populations exposed to the synthetic medium or saline-diluted broth must reflect the appearance of competence in cells which were not previously competent.

Discussion. Although the competence of

populations of 3 species of *Hemophilus* when diluted 10^4- or 10^5-fold and incubated in the defined medium is comparable to the high degree found when exposed to DNA after incubation of a 1:10 dilution in buffered saline, the function of the individual components of the medium requires further investigation. By analogy with the work of Fox and Hotchkiss(8) on the requirements for the "creation" or "recreation" of competence in pneumococcal populations, the amino acids and bivalent cations of the medium may function in formation of DNA binding sites and in the DNA-fixation process. Albumin, essential for competence in the pneumococcus is not required by *Hemophilus* under the conditions investigated.

Whether the components of the medium are required for synthesis of specific receptor sites, for a pinocytotic-like means of incorporation of the DNA molecule, or for active transport of DNA across the cell membrane cannot now be answered. However, demonstration of the emergence or expression of competence in the medium provides a tool for further study of both the emergence and decay of the competent state under chemically defined conditions.

Summary. Three species of *Hemophilus*, grown under the method of Goodgal and Herriott, were shown to emerge to a state of competence for transformation to SM-resistance in a chemically defined medium: L-aspartic, L-glutamic acids in buffered saline containing calcium and magnesium ions. The degree of competence of a "standard" culture was enhanced many-fold in 30 to 60 minutes even when diluted 10^5-fold in the defined medium. The emergence of competence in the synthetic environment was temperature dependent and inhibited by chloramphenicol; multiplication was not necessary. Competent populations washed in the defined medium retain competence; in populations which have lost transformability as a result of washing in buffered saline, competence may be restored. Data presented suggest that the competence which emerges reflects the final attainment of the competent state rather than its "restoration" in cells which were competent at an earlier period during growth of the population.

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CCl_4 Poisoning II: Serum Enzymes, Free Fatty Acids and Liver Pathology; Effects of Phenoxybenzamine and Phenergan®.* (27905)

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Carbon tetrachloride liver damage, as demonstrated by alterations in mitochondrial function, increased total liver lipid and necrosis, has been decreased by adrenalectomy, cord transection and administration of blocking agents(1,2). The changes involved in tissue necrosis and lipid deposition may be caused by separate mechanisms, since Phenergan® pretreatment can reduce tissue Ca^{++} imbibition and the efflux of liver enzymes

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