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Genetic Integration in the Heterospecific Transformation of *Haemophilus influenzae* Cells by *Haemophilus parainfluenzae* Deoxyribonucleic Acid

WILLIAM L. STEINHART AND ROGER M. HERRIOTT

Department of Biochemistry, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland 21205

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The in vivo chemical linkage of *Haemophilus parainfluenzae* deoxyribonucleic acid (DNA) with the *H. influenzae* genome has been found to occur at a much higher level than is suggested by the low efficiency of the heterospecific transformation of an antibiotic resistance marker. This linkage, about 60% of the level with homospecific DNA, was found to involve alkali-stable bonding. The amount of host DNA label released (about 60%) was about the same as that released during homospecific transformation. Also, over 60% of the *H. influenzae* cells adsorbing *H. parainfluenzae* DNA could not form colonies upon plating. This lethality of the heterospecific transformation was not immediate but followed considerable metabolic activity of the host cells. These data are presented to show that the "limited-pairing" hypothesis may be only a partial explanation for the low efficiency of heterospecific transformation. Another hypothesis is presented which takes into account the lethal effect of this kind of transformation.

The heterospecific transformation frequencies among various species pairs in the *Haemophilus* genus are known to be very low relative to homospecific transformation (3, 12). For example, in the case of the transformations between *H. influenzae* and *H. parainfluenzae*, no genetic marker has been found which transforms at a frequency greater than 1% of the frequency of transformation within a single species.

Ten years ago Schaeffer (12) presented the imperfect-pairing hypothesis whereby the heterospecific transformation frequency was considered to be solely a function of the degree of homology between donor and recipient deoxyribonucleic acid (DNA). According to this hypothesis, the decreased frequency of heterospecific transformation was a direct result of a decreased level of integration of donor information. Other investigators (11) have stated that limitations in pairing and subsequent integration are the principal cause of inefficiencies in these transformations. In several reports (4, 6, 8, 12), it has been suggested that the "genetic relatedness" defined by relative heterospecific transformation frequencies be considered a criterion in problems of bacterial taxonomy.

After the presentation of Schaeffer's imperfect-pairing hypothesis, J. M. Thoday suggested a lethality of heterospecific transformation as a possible interpretation of Schaeffer's data (12). This suggestion arose from Thoday's experience with *Drosophila* matings, in which low viability often appeared in genetic transfer between two populations.

In this investigation, experiments were designed to characterize more precisely the integration process during heterospecific transformation by use of biophysical as well as genetic methods. Evidence is presented which suggests that the imperfect-pairing scheme may play only a partial role in inhibiting the transformation of *H. influenzae* by *H. parainfluenzae* DNA, and that the role of induced lethality, as proposed by Thoday, is significant.

MATERIALS AND METHODS

Methods for competence development in *H. influenzae* cultures and for radioactivity assays and sucrose gradient zone sedimentation analysis have been reported in the accompanying paper (14).

Isolation of DNA. The method for purification and shearing of DNA from *H. parainfluenzae* (wild type

P^+ or streptomycin resistant) was precisely the same as the method we used previously for DNA from *H. influenzae* (14). Stocks of *H. parainfluenzae* were generously provided by Y. C. Hsu and S. H. Goodgal. *Escherichia coli* DNA was isolated essentially by the method of Marmur (6). *Bacillus subtilis* 186 DNA was a gift of P. C. Huang.

Tritium labeling of DNA. For the preparation of purified *H. influenzae* ^{3}H-DNA, cells were grown in the defined medium M-Ic (*in preparation*) containing about 25 μc of ^{3}H-thymidine per ml (about 16.5 c/mmole; New England Nuclear Corp., Boston, Mass.). Cells were harvested and washed at a concentration of 10^9 cells/ml; then they were processed for DNA purification. Specific radioactivities of up to 10^6 counts per min per μg of DNA could be obtained in this way. Labeling of *H. parainfluenzae* DNA was obtained in the same way, except that the growth medium consisted of a 4 to 1 mixture of M-Ic medium and Heart Infusion Broth (Difco). Labeling of *E. coli* (*thy⁻ his⁻ ura⁻*) DNA was simplified by the thymidine auxotrophy. To the Maaløe-Hanawalt partially supplemented medium (5), we added 25 μc of ^{3}H-thymidine per ml and 1.5 μg of unlabeled thymidine per ml. The culture was allowed to grow to stationary phase before the DNA was harvested and extracted.

Biological assay of DNA in sucrose gradient fractions. The assay of radioactivity in gradient fractions was described in the accompanying paper (14). The biological assay for DNA in these fractions was a measurement of the transforming activity of antibiotic resistance markers in the *H. influenzae* DNA sedimented. Alternate half-fractions not used in the radioactivity assay were collected into empty scintillation-counting vials. An excess of competent wild-type (Rd^+) cells was added to each vial in a 0.5-ml volume. These transformation mixtures were incubated at 37 C, diluted 1:20, and plated to assay transformants by the agar-overlay technique.

RESULTS

Loss of host genomic label from H. influenzae after uptake of H. parainfluenzae DNA. In experiments reported in the preceding paper (14), it was found that homospecific transformation induced a loss of radioactive fragments from competent cells bearing a tritium label in the DNA. This label release was over and above the release from a control culture which adsorbed nontransforming DNA or no DNA.

In a similar experiment, the effect of heterologous *H. parainfluenzae* DNA on the release of radioactivity from competent, labeled *H. influenzae* cells was determined. On the basis of transformation frequencies, one would not expect to see much of a difference between the host DNA label released from control cultures receiving no DNA or unrelated DNA and that from the heterospecifically transformed culture. This expectation is based on two assumptions: (i) an increased rate and quantity of ^{3}H liberation relative to the control system is recombination-specific, and (ii) the low level of heterospecific transformation is a direct consequence of infrequent recombination. The low efficiency of heterospecific transformation relative to homospecific transformation (at most 1%) was confirmed in this system with DNA from str_{2000} *H. parainfluenzae* (resistance to 2,000 μg of streptomycin per ml), kindly supplied by Y. C. Hsu, and with competent Rd^+ *H. influenzae* cells.

Surprisingly, *H. parainfluenzae* DNA stimulated a release of host DNA label (above background) of more than 50% of that released by homospecific DNA (Fig. 1). In the run represented here, the *H. parainfluenzae*-induced release rate and maximal quantity were about 60% of the level in the homospecifically transformed case, with the time course apparently identical in both cases. Other runs produced a relatively level range of 55 to 61%. If the label-release phenomenon is indeed recombination-specific, these results indi-

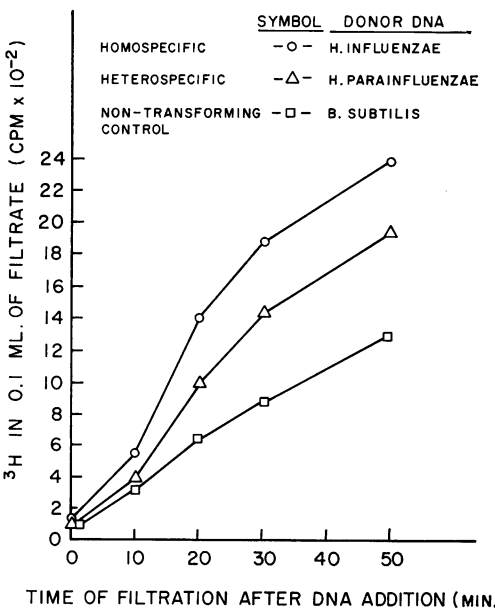

FIG. 1. *Release of radioactive fragments from ^{3}H-thymidine-labeled H. influenzae after adsorption of cold H. parainfluenzae DNA. Three portions of a recipient H. influenzae culture containing ^{3}H-labeled DNA were allowed to adsorb homospecific, heterospecific (H. parainfluenzae), or nontransforming (B. subtilis) donor DNA at 1 μg/ml. The cultures were incubated at 37 C, and deoxyribonuclease (2 μg/ml) was added after 10 min of uptake. At various times after addition of donor DNA, samples of each culture were filtered. ^{3}H levels in filtrate samples were determined as the measure of released host DNA products.*

cate that far more heterospecific integration occurs than is suggested by the low transformation efficiencies.

Heterospecific integration and its stability. A determination of the actual extent of heterospecific integration relative to homospecific integration was undertaken. Lysates were prepared from competent, streptomycin-resistant *H. influenzae* cultures which adsorbed purified, sheared, ^{3}H-labeled donor DNA from *H. influenzae*, *H. parainfluenzae*, or *E. coli*. Preliminary experiments confirmed the equal uptake efficiency of DNA from various sources. To analyze the cold recipient DNA and the integrated and nonintegrated irreversibly adsorbed donor ^{3}H-DNA, sucrose gradient zone sedimentation was employed. The sheared, nonintegrated donor molecules were easily separated from cold recipient DNA by size differences. Integrated donor molecules were defined as those sedimenting with the peak of large recipient molecules with the following control system employed for calculation of amounts of DNA actually integrated in high molecular weight segments. In the control in which *E. coli* ^{3}H-DNA was adsorbed, the small amount of ^{3}H sedimenting with recipient DNA probably did not result from real integration but from an anomalous process of occlusion by the recipient DNA or from random incorporation of some ^{3}H-labeled donor degradation products. It was presumed that this pseudointegration occurs with any donor DNA, and so the amount of pseudointegration with *E. coli* DNA was subtracted from the integration levels of *H. influenzae* and *H. parainfluenzae*. It should be mentioned that this correction factor was a small one and could have been neglected. The subtraction followed corrections for specific radioactivity differences in the various types of donor DNA.

The physical integration level of the heterospecific donor relative to the homospecific donor ranged between 48 and 78%. The sedimentation patterns from one of three runs are shown in Fig. 2. The proportion of integrated to nonintegrated donor molecules appears quite low in these analyses; that is, the cells have adsorbed far more DNA than is integrated. Since very high donor DNA concentrations were used in the initial transformation and since no deoxyribonuclease digestion was included prior to filtration-washing, much of the material in the nonintegrated donor DNA peaks may represent molecules which are not specifically bound by the competent cells or molecules which are bound but not penetrated. The magnitude of these peaks may have been artifactitiously expanded. The stability of the linkage in each lysate was determined by alkali denaturation followed by alkaline gradient sedimentation. In the alkaline gradient patterns presented (Fig. 3), it was found that the integration of *H. parainfluenzae* ^{3}H-DNA into the *H. influenzae* genome included an alkali-stable, covalent bonding. For the sake of clarity, recipient DNA was not plotted in Fig. 3; it peaked precisely at the location of the "integrated" *H. influenzae* and *H. parainfluenzae* DNA peaks. The nearly com-

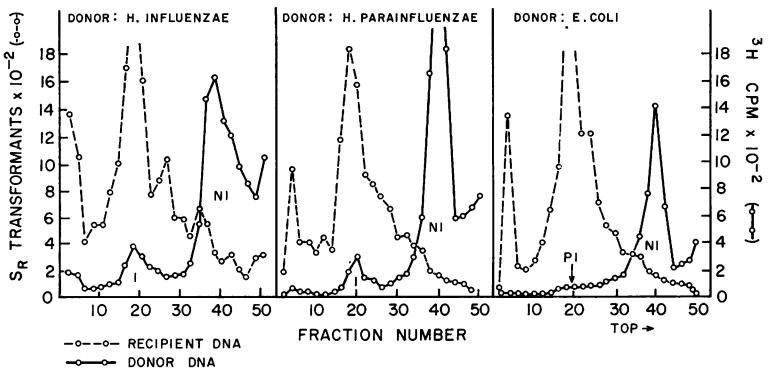

FIG. 2. *Neutral sucrose gradient assay of heterospecific integration. Biologically labeled* str_{2000} *(Sr) competent H. influenzae cultures adsorbed either homospecific, heterospecific (H. parainfluenzae), or nontransforming (E. coli)* ^{3}*H-labeled donor DNA of high specific radioactivity. Donor DNA was added at 5 μg/ml. Cultures were incubated with the donor DNA at 37 C for 40 min, allowing maximal development of linkage. No deoxyribonuclease was used. The cultures were washed and suspended in standard saline-citrate at a concentration of* 10^9 *cells/ml and were lysed with 0.1% (w/v) sodium lauryl sulfate. Lysates were heated to 60 C for 10 min, cooled to 37 C, and treated with Pronase (100 μg/ml) for several hours. Shortly after, samples containing 1 μg or less total DNA were subjected to sedimentation analysis on 5 to 20% neutral sucrose gradients. Centrifugation was carried out for 150 min at 25,000 rev/min and 24 C. I = integrated, NI = nonintegrated, and PI = pseudointegrated donor DNA peaks.*

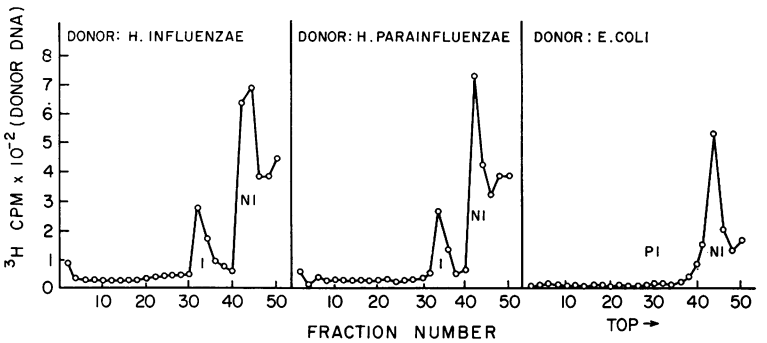

FIG. 3. *Alkaline sucrose gradient assay of covalent heterospecific integration. DNA was denatured with 0.3 N NaOH in samples of the same lysates used in the neutral gradient assays of integration (Fig. 2). Samples of 1 μg or less of DNA were sedimented on alkaline gradients (5 to 20% sucrose in 0.9 M NaCl and 0.1 N NaOH). Sedimentation was carried out for 210 min at 25,000 rev/min and 24 C. Donor ^{3}H-DNA was assayed by radioactive counts. Integrated (I), nonintegrated (NI), and pseudointegrated (PI) peaks are indicated.*

plete absence of the *E. coli* pseudointegration background under alkaline conditions means that random incorporation of the donor label is not responsible for the minor pseudointegration in neutral gradients. More likely, a weak, nonspecific binding is responsible. The higher figures (about 70%) for the *H. parainfluenzae* to *H. influenzae* integrating proportion came from the alkaline gradient analyses in which the integrated donor peaks were better defined and the pseudointegration background was almost nonexistent. These higher values are considered more realistic.

Lethal effect of heterospecific transformation. During the preparation of lysates for the integration efficiency analyses described above, it was noted that uptake of *H. parainfluenzae* DNA by highly competent *H. influenzae* cells always led to a decreased viability of the recipient culture. To determine the extent of this lethality, competent str_{2000} *H. influenzae* cells were suspended in the nongrowth competence medium M-II (13) at a concentration of 10^9 cells/ml and the suspension was divided into several portions. Purified DNA from novobiocin-resistant *H. influenzae* and purified DNA from wild-type *H. parainfluenzae* were added separately to two cultures at a saturating concentration of 1 μg/ml. Control cultures received an equivalent volume of saline or the *H. parainfluenzae* donor DNA previously digested with 2 μg of deoxyribonuclease per ml for 15 min at 37 C. After incubation for 30 min at 37 C, all of the cultures were diluted and plated in triplicate for a cell viability assay. Competence was estimated from the $Nov_{2.5}$ transformants (resistance to 2.5 μg of novobiocin per ml) in the culture receiving DNA from $Nov_{2.5}$ *H. influenzae*. In these studies, a lethal event was the failure of a host cell to form a colony upon plating. We found that 60 to 70% of the fully competent recipient cell population was killed after incorporation of heterospecific DNA (Table 1). It should be noted that all cells took up DNA; i.e., every cell was competent as determined by the method of Goodgal and Herriott (2). The lethal effect decreased when the competence, and therefore the number of molecules adsorbed, decreased. Deoxyribonuclease-digested *H. parainfluenzae* DNA had no lethal effect, confirming that it is indeed the intact DNA in the preparation which kills. Homospecific DNA may have a lethal effect; however, it is just at the edge of the normal variance, so that this effect cannot be confirmed. *E. coli* DNA appears to have a killing power similar to that of the homospecific DNA, no effect being present when the DNA is digested. These latter cases require further investigation, since, in the case of *E. coli* DNA, integration is not involved.

In testing the possibility of host cell lysis during the heterospecific transformation, an extremely low level of host-transforming str_{2000} DNA was found free in the medium. Excluding the possibility of degradation, the presence of this extremely small amount (10^{-4} μg/ml) of DNA suggested that significant cell lysis does not occur during the incubation period. Also, observation of the doomed population with a phase-contrast microscope indicated that aggregation of cells induced by exposure to heterospecific DNA was not responsible for the apparent viability drop.

Limited growth of doomed cells. The lethality of the *H. parainfluenzae* DNA demonstrated in the previous section cannot be attributed to any particular step since the criterion was merely colony formation. If the effect is one of immediate toxicity, one should see an immediate drop in

TABLE 1. *Lethality of heterospecific transformation (loss of colony-forming capacity) in competent H. influenzae cultures adsorbing H. parainfluenzae DNA*

Donor source	Day[a]	Plate count	Avg viable titer	Fraction of cells killed (per cent of saline control)
Saline (control)	1	208, 212, 208	10.4×10^8	
	2	209, 203, 209	10.3×10^8	
		188, 210, 197	9.9×10^8	
	3[b]	248, 216, 246	11.8×10^8	
H. parainfluenzae (deoxyribonuclease-treated control)	1	222, 224	11.2×10^8	
H. influenzae (homospecific)	1	164, 156, 161	8.0×10^8	24
	2	176, 165, 183	8.8×10^8	11–14
	3[b]	256, 200	11.4×10^8	3
H. parainfluenzae (heterospecific)	1	76, 80, 69	3.8×10^8	64
		83, 86, 79	4.2×10^8	60
	2	64, 69, 70	3.4×10^8	66–67
		66, 68, 53	3.1×10^8	69–70
	3[b]	168, 170, 183	8.7×10^8	26
		161, 174, 173	8.5×10^8	28
E. coli (nontransforming)	1	214, 180, 168	9.4×10^8	10
		212, 182, 196	9.8×10^8	6

[a] Plate counts were done on different days, with one or two runs assayed in triplicate.
[b] Recipient cells had limited competence, about 1/3 the normal level.

metabolic activity. If it is a strictly genetic effect, the synthetic steps might continue for at least a generation. To test these possibilities, two cultures of competent *H. influenzae* (5×10^8 cells/ml in growth medium) were exposed to no DNA and to excess *H. parainfluenzae* DNA, and their turbidity as a measure of growth was followed as the cultures were aerated. Both cultures continued to "grow" (become more turbid) through a time period corresponding to 1.5 generations (Fig. 4). The inflections at time periods of 60 to 70 min and 100 to 103 min represent equal dilution of the cultures to maintain them in late log phase. Lethality appeared to be expressed at about 60 min after addition of the growth medium. After about 90 min, multiplication of the survivors was evident and their growth pattern mimicked that of the control culture, except that the absolute concentrations were displaced downward to an extent, in fine agreement with the proportion of doomed cells determined by plating in the previous experiments. The drop in the optical density of the transformed culture after 70 min suggested lysis of a fraction of the cells. This lysis may have been induced by the dilution after 60 min and may suggest a weakness of the doomed cells in terms of resistance to environmental shock. Perhaps the time course of the expression of lethality indicates that the doomed cells cannot divide but are able to expand to a maximal size. Whatever the situation, considerable metabolic activity followed the lethal uptake of *H. parainfluenzae* DNA.

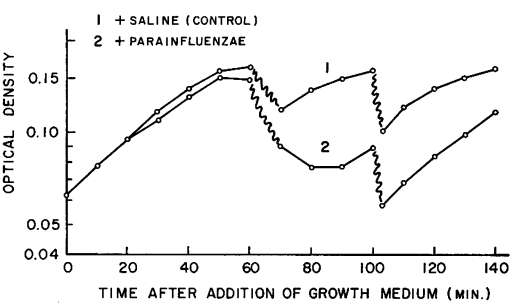

FIG. 4. *Lethality of heterospecific transformation: limited growth after DNA uptake. A competent H. influenzae culture in M-II (nongrowth) medium was divided, one-half receiving 1 μg of H. parainfluenzae DNA per ml, the other receiving saline. After 30 min at 37 C, Heart Infusion Broth plus nicotinamide adenine dinucleotide was added to both cultures to allow growth and to dilute the cultures to a log-phase concentration. Incubation was continued for 140 min at 37 C. Optical densities of both cultures were measured at λ = 650 nm every 10 min in the sidearm of the culture tube. Simultaneous dilutions of each culture were made as the cells approached the stationary-phase concentration.*

DISCUSSION

We have developed a hypothesis which explains the major cause of the low frequency of hetero-

specific transformation in the *H. parainfluenzae-H. influenzae* system. This hypothesis is based on the correlation of data from the three groups of experiments presented here which describe the behavior of *H. parainfluenzae* DNA inside the *H. influenzae* cell. Covalent linkage of *H. parainfluenzae* DNA to the *H. influenzae* genome reaches 48 to 78% of the level of homospecific integration. A similar proportion (55 to 61%) of label is released from the tritiated *H. influenzae* genome, and, again, a similar proportion (60 to 70%) of the *H. influenzae* culture is doomed by the heterospecific transformation. Although all of the cells adsorb *H. parainfluenzae* DNA, perhaps only part (48 to 78%) of the population integrates as much *H. parainfluenzae* DNA as it would *H. influenzae* DNA. Or it may be that all of the cells integrate 48 to 78% as much *H. parainfluenzae* DNA as *H. influenzae* DNA. Or perhaps the situation is somewhere between these two possibilities. At present, we cannot determine which is the case. Separation of the doomed from the surviving cells would allow us to determine whether it is primarily the doomed cells which integrate the heterospecific DNA. To date, no method of separation has been found.

We propose that the fraction of the *H. influenzae* population integrating heterospecific DNA is slightly greater than the fraction killed by this activity, and that only a fraction of those few integrating cells which escape death are transformed with respect to the particular marker examined. Thus, it is tempting to suggest that this is an example, in bacteria, of the inefficiencies of interspecific genetic mixtures found in higher organisms, as mentioned many years ago by Thoday. Note that "inhibited pairing" may retain some importance, however, since the relative integration level in the heterospecific transformation is only 48 to 78%, not 100%.

Sucrose gradient sedimentation allowed only a rough estimate of the extent of heterospecific integration, but the values obtained for *H. parainfluenzae* DNA are clearly far greater than those suggested by the transformation frequencies. Indeed, the low efficiency of heterospecific transformation does not provide direct evidence that little integration occurs. On the basis of sedimentation analyses, Pène and Romig (9) found that *B. cereus* DNA does not integrate into the *B. subtilis* genome. This situation also involves a heterospecific DNA uptake, but the *B. cereus* DNA is "genetically inert," that is, there is no heterospecific transformation. The differences between these interspecific systems probably involve a taxonomic separation problem. The *B. cereus-B. subtilis* system may resemble a heterogeneric situation more than a heterospecific one in terms of genetic transfer. A lack of homology between DNA from *B. cereus* and DNA from *B. subtilis* has been noted in studies of hybridization ability between single strands (8). From these studies, it was concluded that *B. cereus* and *B. subtilis* are not "genetically related." Hybridization ability between *H. parainfluenzae* and *H. influenzae* single strands remains to be determined; the base content is the same in the two species of DNA

There is a striking quantitative similarity between the integration and host label release data. Acceptance of our conclusion on heterospecific integration in this system supports acceptance of the label release phenomenon reported in the preceding paper (14) as truly recombination-specific. The use of a nonintegrating, heterogeneric DNA in control sedimentations rules out the possibility of pseudointegration of *H. parainfluenzae* DNA. S. H. Goodgal (*personal communication*) has recently indicated that he has also observed extensive integration of *H. parainfluenzae* DNA into the *H. influenzae* genome.

The killing effect observed in these studies is quite unlike that in streptococcal transformation (10); in streptococcal transformation, uptake of DNA from widely variant, including homospecific, sources has the same lethality. In the *Haemophilus* system, only the donor of "limited heterology" was found to bear a severe lethality. DNA from other strains and species in this genus are presently being studied for their lethal and integration capacities. Any case for homospecific donor lethality is weak but bears further investigation. Schaeffer and other investigators studying the *H. parainfluenzae-H. influenzae* system may not have observed the killing effect because of the lower level of competence of their cells. Schaeffer's frequencies were 10- to 50-fold lower than ours for similar antibiotic resistance markers. A dose-response curve for the lethal effect of *H. parainfluenzae* DNA is presently being developed. Also, transformation under conditions which allow growth can obscure the effect. After 1.5 generations there would be an initial 65% reduction in survivors; this reduction would probably be lost in random error variability of the plating assay.

The lethality mechanism remains unresolved. The fact that the doomed cells in liquid culture increase in mass through more than a generation may indicate that the mortal effect involves synthesis of materials, coded for by the integrated *H. parainfluenzae* DNA, which are obstructive to the *H. influenzae* cell.

In light of the present results, it is somewhat puzzling that in earlier work Cabrera and Herriott

(1) were unable to cause lethal transformations with ultraviolet-inactivated homologous DNA molecules Tests for physical integration in these systems are needed, and if integration does not take place it would be of interest to learn what changes block the integration steps.

ACKNOWLEDGMENTS

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