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Mechanism of Homospecific DNA Uptake in *Haemophilus influenzae* Transformation

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Summary. DNA uptake by competent *H. influenzae* cells requires the presence of a specific base sequence (uptake site) on the entering DNA duplex. This sequence is probably recognized by a receptor on the cell surface. We have examined the kinetics and stoichiometry of DNA uptake by competent cells and have shown that the results are consistent with a simple model involving: 1) reversible binding of the DNA uptake site to a cell surface receptor, 2) an irreversible step resulting in a commitment toward DNA uptake, and 3) transport of the DNA duplex into the cell. We have also shown that a competent *H. influenzae* cell can absorb only 4 to 8 molecules of DNA, regardless of their length. To explain this counting mechanism, we suggest that each cell has only 4 to 8 receptors and that each receptor can be used to transport only one molecule of DNA.

Introduction

Transformable bacteria have the ability to acquire a heritably altered phenotype by taking up DNA from the external medium and incorporating it into their genome (for reviews, see Notani and Setlow, 1974; Lacks, 1977). Several species of bacteria utilize this means of genetic exchange. Our studies are with *Haemophilus influenzae,* a gram negative organism with very efficient transformation ability. Greater than 99% of a cell population can be "induced" to a competent state by transferring log phase cells from a rich medium into a defined, non-growth medium which allows continued protein synthesis (Herriott et al., 1970). Donor DNA is considered to be taken up (irreversibly bound) when it becomes resistant to nuclease treatment and cannot be eluted from the cells by high salt washes. After uptake, the donor DNA is efficiently integrated into the genome to yield transformants (Notani and Goodgal, 1966).

Recently, we have improved our understanding of the mechanism by which competent *H. influenzae* cells take up DNA. Scocca et al. (1974) have shown that these bacteria will only take up *Haemophilus* DNA; foreign DNAs are taken up poorly and do not compete with *Haemophilus* DNA. Sisco and Smith (1979) have shown that this uptake specificity depends on the recognition of a specific DNA sequence (uptake site) in *Haemophilus* DNA and that this sequence is distributed in about 600 copies over the genome. A membrane-bound cell surface protein appears to act as a receptor in recognizing the DNA sequence (Deich and Smith, unpublished observations). These findings suggest a simple model for DNA uptake (Fig. 1). The first step (recognition) is the initial interaction between the DNA uptake site and the receptor. This step may be regarded as a simple, reversible, DNA-protein interaction analogous to the binding of *lac* or λ-repressor to DNA. The second step is an irreversible initiation event which results in a commitment toward DNA uptake. The final step is the transport of the DNA duplex into the cells. This model is similar in outline to the three-step model proposed by Fox and Hotchkiss (1957) and Lacks (1977) for DNA uptake in pneumococcal transformation, although the molecular mechanisms by which the steps occur are apparently quite different. The experimental results presented in this paper support the proposed model and enable us to estimate some of the kinetic and stoichiometric parameters of the uptake mechanism.

Materials and Methods

1. Bacterial Strains. The following sublines of *H. influenzae* Rd were used: KW20 (wild type); KW21 (EryR); KW35 (StrR, NovR)

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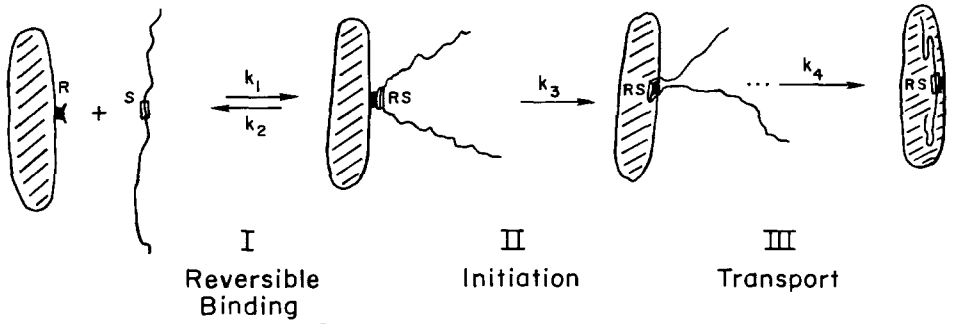

Fig. 1. Proposed model for DNA uptake by competent *H. influenzae* cells. *I.* Reversible binding. In this step, DNA uptake sites (*S*) are reversibly bound to transformation receptors (*R*). This binding can be considered a simple, reversible DNA-protein interaction analogous to lac or λ repressor binding to DNA. Both uptake sites and receptors are free to exchange any number of times. *II.* Commitment to DNA uptake. In the second step, the uptake site and receptor form a complex which is committed to DNA uptake. While the DNA duplex is still outside the cell (and presumably still DNAse sensitive), the components of the uptake site-receptor complex are no longer exchangeable. Such non-exchanging complexes have been demonstrated in pneumococcus transformation (Seto and Tomasz, 1974; Lacks and Greenberg, 1976) but not in *Haemophilus*. It is possible that this non-exchanging complex may only exist as an intermediate step in DNA uptake. *III.* Transport. As the final step, the DNA duplex is converted into a DNAse resistant non-elutable form. We define this process as irreversible binding. For simplicity, we have assumed that the DNA is transported into the cell, although this has never been formally demonstrated

(Wilcox and Smith, 1975). The EryR, StrR, and NovR markers confer resistance to erythromycin (10 μg/ml), streptomycin (200 μg/ml), and novobiocin (5 μg/ml).

2. Cell Growth and Competence Development. H. influenzae cells were grown at 37° C in either 3.7% brain heart infusion (BHI, Difco), or 2.5% heart infusion (HI, Difco), supplemented with 2 μg/ml nicotinamide adenine dinucleotide (Sigma) and 10 μg/ml Hemin (Sigma). The media are referred to as sBHI and sHI, respectively.

For competence development, cells were grown to a density of 5×10^8 cells/ml in sHI and transferred to MIV medium, a complex synthetic medium designed by Herriott et al. (1970) for consistently producing high levels of competence. After incubation in the MIV medium at 37° C for 100 min, cells were either used directly or made 18% in glycerol and frozen at −70° C for future use.

3. DNA Preparation. Unlabelled *H. influenzae* marker DNA was prepared from cells grown to late log phase in sBHI. ^{32}P-labelled *H. influenzae* DNA was prepared from cells grown in low-phosphate sHI (Scocca et al., 1974) containing 30 μCi/ml [^{32}P]orthophosphate (NEN; carrier free). DNA was extracted by the Marmur (1961) procedure.

Sheared DNA was prepared by three passes through a #21 or a #25 gauge needle and the molecular weight was determined by sucrose gradient velocity centrifugation (Burgi and Hershey, 1963). Sonicated DNA was prepared by a single burst of 5 s at the No. 5 position on a Branson Sonifier and molecular weight was determined by electrophoresis on 1% agarose gels (McDonell et al., 1977).

4. DNA Uptake and Transformation. Competent cells were suspended in MIV medium (approximately 5×10^8 cells/ml) at 37° C and transforming DNAs were added. Uptake reactions were allowed to proceed for 10 min unless otherwise noted.

For transformation assays, cell suspensions were serially diluted in sBHI and plated with 10 ml of 1% agar in sBHI. Plates were incubated for 90 min at 37° C to allow expression of the transformed phenotype and then overlayered with 10 ml of sBHI-agar containing the appropriate antibiotic (200 μg/ml streptomycin or 10 μg/ml erythromycin).

To measure irreversibly-bound, radioactively-labelled DNA, two procedures were used. For fast kinetic studies, uptake was stopped by 1:1 dilution of the cell suspension into tubes containing ice-cold DNAse I (Sigma) solution (100 μg/ml of enzyme in 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$). In the other experiments, DNA uptake was allowed to go to completion and unabsorbed DNA was digested by the addition of DNAse I to 50 μg/ml. In all cases, cells in suspension were incubated on ice for five minutes in the DNase mixture, made 0.5 M in NaCl, centrifuged for 1 min in an Eppendorf 3200 centrifuge, and washed once with MIV medium plus 0.5 M NaCl. Uptake of radioactive label was determined either by Cerenkov radiation (about 30% efficienty for ^{32}P) or by counting in Triton X-100 toluene scintillation fluid (Patterson and Greene, 1965).

Results

1. DNA Absorption Depends Only on Uptake Site Concentration. Sisco and Smith (1979) have demonstrated that competent *H. influenzae* cells are able to discriminate between *Haemophilus* DNA and other DNAs during uptake because they recognize a specific base sequence which occurs about 600 times in the *Haemophilus* genome. This result suggests that there is a receptor on the surface of competent cells which specifically binds this sequence to initiate DNA uptake. Such a receptor has tentatively been identified in membrane preparations from competent *H. influenzae* cells (Deich and Smith, unpublished observations).

This interaction between uptake site and receptor explains why absorption of *Haemophilus* DNA by competent cells is nearly independent of the concentration of competing DNAs which lack the site. It also predicts that the efficiency of binding for each uptake site should not depend on the length of the

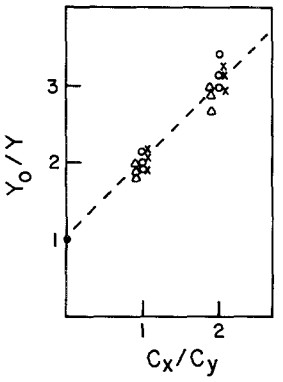

Fig. 2. Effect of DNA length on uptake competition. Unlabelled *H. influenzae* DNA of various lengths was prepared by shearing or sonication as described in *Methods*. 1 to 2 μg of each of these DNAs was added to an individual 1 ml reaction mixture containing 5×10^8 competent cells and 1 μg [^{32}P]*H. influenzae* DNA. The uptake reactions were allowed to proceed for 10 min at 37° C and the amount of irreversibly bound labelled DNA was determined (see Methods). The data is plotted as the ratio of label absorbed in the absence of cold competing DNA (Y_0) to the label absorbed in the presence of competing DNA (Y) versus the weight ratio of cold competing DNA (C_x) to labelled DNA (C_y). We have previously shown that a slope of one (dashed line) corresponds to equal weight/weight competition on such a plot (Deich and Smith, 1979). o---o, control DNA (50 kb avg.); △--△, sheared DNA (20–25 kb); ×---× sonicated DNA (0.8–1.5 kb)

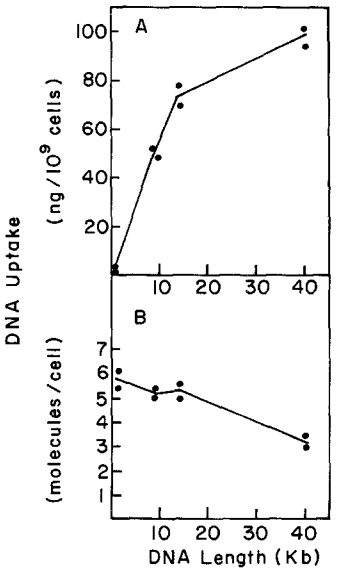

Fig. 3A and B. Effect of DNA length on uptake in competent *H. influenzae* cells. [^{32}P]*H. influenzae* DNA of various lengths was prepared and sized as described in *Methods*. One μg of each length DNA was incubated with a 1 ml suspension of competent cells (5×10^8/ml) in MIV medium and irreversible DNA uptake determined (see Methods). **A** Total DNA uptake *vs* average DNA length (kb). **B** Molecules of DNA taken up per cell *vs* DNA chain length

DNA molecule to which it is attached. To test this prediction, we have compared the ability of unlabelled *Haemophilus* DNA preparations of varying length (1 to 50 kb) to compete with ^{32}P-labelled *Haemophilus* DNA for uptake into competent cells. The data are plotted in Fig. 2 as the weight ratio of cold competing DNA to ^{32}P-labelled DNA (abscissa) versus the ratio of the label absorbed in the absence and presence of cold competing DNA (ordinate). We have previously shown that such a plot will yield a straight line whose slope is equal to the ratio of the binding efficiencies of the competing DNAs (unlabelled and labelled) (Deich and Smith, 1979). In all cases, our experimental points fall approximately on a straight line with a slope of one, indicating that the binding of *Haemophilus* DNA uptake sites to competent cells is independent of DNA length from 1 kb to 50 kb. In the following section, we demonstrate that smaller DNAs are taken up by the cells and do not act by simply blocking receptors.

2. Competent Cells Absorb a Fixed Number of DNA Molecules. Two mechanisms might limit the amount of DNA absorbed by competent cells. The first might limit the *number* of DNA molecules absorbed. This could be accomplished by using up the receptor stoichiometrically; for example, by its entering the cell along with the uptake site. In this case, each receptor could initiate transport for only one DNA duplex. The second mechanism might limit the *mass* of DNA absorbed; for example, by exhaustion of some cofactor required for transport of a given length of DNA. These two possibilities may be distinguished experimentally by exposing competent cells to labelled DNAs of different lengths. In the first case, one would expect the amount of label absorbed to vary directly with DNA length; in the second, it should remain unaffected by varying length.

Competent cells were fed [^{32}P]*Haemophilus* DNA of average lengths varying from 1 kb to 40 kb prepared by shearing or sonication. A typical result of such an experiment is shown in Fig. 3. The amount of label taken up by competent cells is proportional to the length of the labelled transforming DNA. On the other hand, the total number of molecules taken up per cell is nearly constant, showing a less than twofold change over this wide range of DNA lengths. The number of molecules absorbed per competent cell varies somewhat between different cell preparations but generally lies in the range of 3 to 8. This result suggests that there is a discrete number of receptors on the surface of competent cells and that each receptor acts only once. We cannot rule out the possibility that fewer receptors are used a fixed number of times. However, Stuy and Stern (1964) have demonstrated that the number of receptors per compe-

tent *H. influenzae* cell is greater than or equal to two.

3. Irreversible Commitment to DNA Uptake. DNA binding to competent cells can normally be divided into three steps: an initial reversible binding to the receptor, irreversible formation of a DNA-receptor complex (i.e., a non-exchanging complex which is committed to DNA uptake), and transport of the DNA into the cell. In the case of pneumococcus, an irreversibly committed DNA-receptor complex can be demonstrated in the presence of K^+ and EDTA (Seto and Tomasz, 1974) or in certain uptake-deficient mutants (Lacks and Greenberg, 1976). In *Haemophilus,* such a DNA-receptor complex has never been demonstrated under conditions that are not permissive for DNA uptake. It seems probable that in DNA binding to competent *Haemophilus* cells, initiation of transport is itself the first "irreversible" step.

If our simple model of DNA uptake is correct, we can determine both the rate of the formation of committed DNA-receptor complexes (k_3) and the dissociation rate of the uptake site-receptor complex (k_2). To do this, we perform a "marker challenge" experiment in which competent cells are initially fed an amount of transforming DNA sufficient to saturate their uptake system ($V \sim V_{max}$), and later challenged with a second genetically marked DNA.

Since the amount of transforming DNA is saturating, at any given time nearly all receptors should be bound to DNA uptake sites, as either reversibly or irreversibly committed complexes. Hence, the formation of committed DNA-receptor complexes should follow first-order kinetics and the number of reversibly bound receptors should decrease exponentially with a half-time of $\ln 2/k_3$.

Experimentally, we can measure the decrease in the number of reversibly bound receptors by challenging the cells with a second genetically marked DNA. We then compare the number of transformants for the marker to the time of challenge. When this is done (Fig. 4), the number of transformants for the second marker (streptomycin resistance) does, in fact, decrease exponentially with a half-time of 40 ± 5 seconds. This corresponds to a value for k_3 of $1.7(\pm 0.2) \times 10^{-2}\ s^{-1}$. Identical results have been obtained using other marker DNAs.

A certain fraction of receptors will act as if they are irreversibly committed instantaneously. In fact, these are reversibly bound receptor-DNA complexes which become irreversibly committed before DNA exchange can occur. They comprise $k_3/(k_2+k_3)$ of the reversibly bound complexes. Consequently, the apparent number of reversibly bound receptors extrapolated to zero-time (R_0') will be less than the true

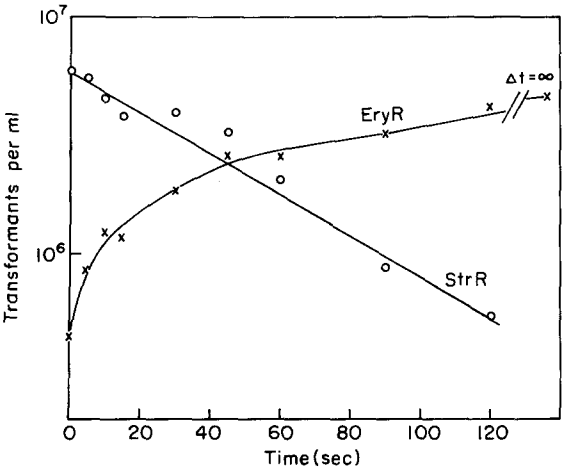

Fig. 4. Marker challenge for transformation in competent *H. influenzae* cells. At time zero, 1 μg/ml of Erythromycin resistant (Ery R) marker DNA was added to a suspension of competent cells (5×10^8/ml) in MIV medium. At intervals of 5–120 s, as shown, 0.5 ml aliquots of this suspension were added to tubes containing 5 μg each of Streptomycin resistant (Str R) marker DNA (10 μg/ml final concentration) and incubated 30 min. For the zero time point, EryR DNA (1 μg/ml) and StrR DNA (10 μg/ml) were added simultaneously to the competent cell suspension. EryR and StrR transformants were determined by plating (see *Methods*). o---o, StrR transformants/ml: ×---×, EryR transformants/ml

number of receptors (R_0) by a factor of $R_0'/R_0 = k_2/(k_2+k_3)$.

The ratio, R_0'/R_0, is identical to the experimentally determined ratio, T_0'/T_0, where T_0' equals the number of transformants for the challenging marker extrapolated to zero challenge time, and T_0 equals the number of transformants for this marker when both transforming DNAs are added simultaneously to the cells. Our results give a value of $T_0'/T_0 \approx 1$, which implies that $k_2 \gg k_3$. Therefore, each receptor must reversibly exchange DNA molecules a number of times on the average before the irreversible commitment occurs.

4. Kinetics of DNA Uptake. The rate of DNA absorption (irreversible uptake) by competent *H. influenzae* cells was examined over a range of DNA concentrations from 50 to 1,000 ng/ml (Fig. 5). When DNA is in excess, absorption proceeds rapidly to a saturation level of 120 to 140 ng DNA/10^9 cells and is complete within 5 min. When DNA is limiting, a similar rapid uptake of 30–40% of the DNA is followed by a much slower absorption of the remaining DNA which continues for over 30 min or until the cells reach the saturation level. The initial rate of DNA uptake increases with DNA concentration to a limiting value of approximately 2.0 ng/s for 10^9 cells in 1 ml at approximately 500 ng/ml DNA. According to our model for DNA uptake, Fig. 5 can be re-

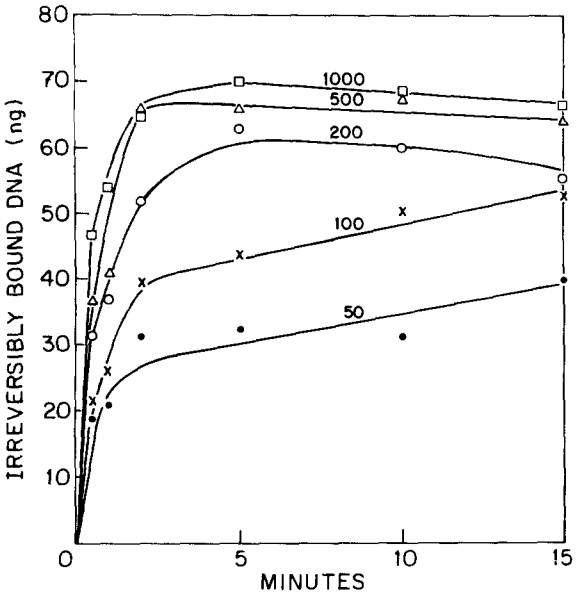

Fig. 5. Kinetics of DNA uptake by competent *H. influenzae* cells. [^{32}P] *H. influenzae* DNA was added at concentration of 50 to 1,000 ng/ml suspensions of competent *H. influenzae* cells (5×10^8/ml) in MIV medium at 37° C. 1 ml aliquots of the reaction mixture were removed at intervals as shown and the uptake reactions stopped by diluting 1:1 with ice-cold DNase I (50 µg/ml) in 10 mM Tris (pH 7.5)+5 mM $MgCl_2$. Irreversibly bound radioactive DNA was determined as described in *Methods*. ●---●---●, 50 ng/ml DNA; ×---×---×, 100 ng/ml DNA; ○---○---○, 200 ng/ml DNA; △---△---△, 500 ng/ml DNA; □---□---□, 1,000 ng/ml DNA

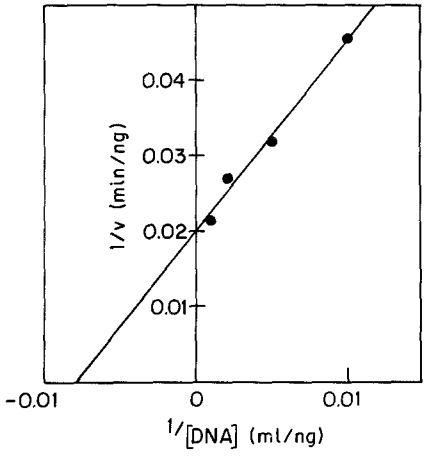

Fig. 6. Lineweaver-Burke plot of DNA uptake in competent *H. influenzae* cells. Reciprocal velocities of DNA uptake determined from the 0.5 min points of Fig. 1 are plotted against the reciprocals of DNA concentration

displayed as a Lineweaver-Burke plot. When this is done for short times (30 s and 60 s), a straight line is obtained (Fig. 6) with a K_m of 100 ng/ml DNA (4×10^{-11} M uptake sites) and a V_{max} of 2.0 ng/s/10^9 cells.

For our DNA uptake model (Fig. 1) $V_{max}=k_3k_4R_0/(k_3+k_4)$, where R_0 is the initial concentration of transformation receptors. Given that each competent cell has 6 ± 3 receptors (see section # 2), that $k_3=1.7\times10^{-2}$ s^{-1} (see section # 3) and that the average length of our transforming DNA is 50 kb (result not shown), we can calculate a value of $k_4=1.5\pm0.5\times10^{-2}$ s^{-1}. This corresponds to a DNA transport rate of 750 ± 250 bp/s at each receptor.

Discussion

DNA uptake by competent *H. influenzae* cells requires recognition at the cell surface of a specific sequence on the donor DNA (Sisco and Smith, 1979). Even a large excess of DNA, if it lacks uptake sites, will not interfere with transforming DNA uptake (Scocca et al., 1974; Deich and Smith, 1979). In this paper, we have demonstrated that the efficiency of uptake-site binding to cells is independent of DNA length when the DNA molecule which contains the site varies over a range from 1 kb to 40 kb. The initial step of the DNA uptake reaction may thus be regarded as a simple, bimolecular interaction between DNA uptake sites and cell-surface transformation receptors.

The uptake of DNA by competent *H. influenzae* cells is consistent with the kinetic model we have proposed; this model is similar to that proposed by Fox and Hotchkiss (1957) and modified by Lacks (1977) for pneumococcal DNA uptake (Fig. 1). The rate constants for non-exchangeable binding (k_3) and uptake (k_4) are substantially greater in *Haemophilus* than in pneumococcus (see Table 1), in agreement with the general observation that DNA uptake in *Haemophilus* is much faster than in pneumococcus.

While the pneumococcus and *Haemophilus* systems can be explained by similar kinetic models, it

Table 1. Transformation constants, *Streptococcus* vs *Haemophilus*

Constant	*S. pneumoniae*	*H. influenzae*
Km [DNA]	40 ng/ml[a]	100 ng/ml (4×10^{-11} M uptake sites)
V_{max} [DNA/10^9 cells]	0.3 ng/s[a]	2.0 ng/s
k_1 [DNA]	0.1 ng^{-1} s^{-1} ml[a]	1.7 ng^{-1} s^{-1} ml [2.5×10^9 M^{-1} s^{-1} uptake sites]
k_2	3.3×10^{-2} s^{-1}[a]	2×10^{-1} s^{-1}[b]
k_3	6.7×10^{-4} s^{-1}[c]	1.7×10^{-2} s^{-1}
k_4 [DNA/receptor]	80 bases/s[c]	500–1,000 bp/s
Receptors/cell	50 (3)	6 ± 3

[a] Fox and Hotchkiss, 1957
[b] From Stuy and Stern, 1964
[c] Lacks, 1977

should be emphasized that the molecular mechanisms of their DNA uptake are quite different. DNA uptake in competent *Haemophilus* requires recognition of a specific base sequence while competent pneumococcus can bind and absorb any DNA. In addition, transforming DNA enters *Haemophilus* cells as intact duplex molecules while in pneumococcus, it undergoes extensive endo- and exonucleolytic processing during uptake (Notani and Setlow, 1974; Lacks, 1977).

The frequency of uptake sites in *Haemophilus* DNA is about one site per 4 kb (Sisco and Smith, 1979); hence, any genetic marker should be within 2 kb of an uptake site (on the average). Since DNA is taken up at a rate of 500–1,000 bp/s, the expectation is that any given DNA marker should enter the cell within 2 to 4 s. Stuy and Stern (1964) demonstrated a lag of 2 to 2.5 s in the uptake of certain drug-resistance markers. This lag could be related to the distance from the markers to the nearest uptake site on the DNA molecule.

The kinetic study of *Haemophilus* transformation by Stuy and Stern (1964) also showed a delay of 4–5 s in marker uptake when competent cells were treated with a marker DNA in the presence of saturating amounts of unmarked DNA. They interpreted this delay as representing the turnover of receptors during uptake. However, our results suggest that receptors do not turn over during uptake, supporting the suggestion by Lacks (1977) that this delay is instead a measure of the rate of reversible DNA exchange at the receptor. The observed delay is consistent with a reversible dissociation rate (k_2) of about 0.2 s^{-1}; this is about 10-fold greater than the irreversible binding rate (k_3) and in agreement with our general observation that $k_2 \gg k_3$.

If we combine this value of k_2 with our previously determined values of K_m, k_3, and k_4 and use the relation $K_m = k_4(k_2+k_3)/[k_1(k_3+k_4)]$ (for derivation, see, for example, Wong, 1975), we can calculate the initial binding rate k_1 to be approximately 2.5×10^9 M^{-1} s^{-1}. By comparison, the binding rates of the *lac* and λ repressor proteins are 5×10^9 M^{-1} s^{-1} and 3×10^{10} M^{-1} s^{-1} respectively (Riggs et al., 1970; Ptashne, 1971). This binding rate seems extremely high since the receptor protein is presumably bound at the cell surface and not freely diffusing in solution. We currently offer no explanation for this. Finally, we can use the reversible rate constants k_1 and k_2 to calculate the in vivo dissociation constant of the uptake site-receptor complex as $K_d = k_2/k_1 = 8 \times 10^{-11}$ M.

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