ON THE TWO-STEP NATURE OF BACTERIOPHAGE ADSORPTION

by

GUNTHER S. STENT* AND ELIE L. WOLLMAN**
California Institute of Technology, Pasadena 4 (U.S.A.)

INTRODUCTION

The rate of adsorption of bacteriophage particles, *i.e.*, the rate at which they are fixed specifically and irreversibly to the surface of host cells, has been found to increase proportionally to the bacterial concentration (SCHLESINGER[1]). In experiments concerning the cofactor activation of T4 bacteriophage we noticed, however, that this relation no longer holds when the number of sensitive host cells per unit volume is raised above a certain limit: there exists a maximum rate of adsorption which cannot be exceeded no matter how dense a bacterial suspension is employed (WOLLMAN AND STENT[2]). On examining the temperature coefficient of the adsorption rate we also observed a strong dependence of this rate on temperature. Since these findings are not in harmony with the simple two-body collision model of bacteriophage adsorption which SCHLESINGER, and later DELBRÜCK[3], used successfully in the interpretation of their observations, we have attempted to extend their analysis.

MATERIALS

Cofactor-requiring T4.38 bacteriophage (WOLLMAN AND STENT[2]) has been used in the experiments here reported. For adsorption of the phage, dilutions of a 24 h culture of *E. coli* grown in F (lactate) synthetic medium at 37° C with aeration, washed and resuspended in DIFCO nutrient broth immediately before use, were employed throughout this work.

MEASUREMENT OF ADSORPTION RATES

The rate of adsorption is measured by first mixing a suspension of virus particles and bacteria and then diluting this mixture after various time intervals in order to separate the phages still unadsorbed from the sensitive cells. The fraction of the phage input adsorbed at the time of the dilution can be estimated, in case of cofactor requiring phages, by plating a dilution of the adsorption mixture directly on cofactor-free agar plates. All those phages adsorbed to cells before the initial dilution of the adsorption mixture will form plaques. The free phages cannot be adsorbed on the cofactor free plates and hence will not register as infective centers (ANDERSON[4]).

* Merck Fellow of the National Research Council; present address: Universitets Institut for Cytofysiologi, Copenhagen Ø (Denmark).
** Fellow of the Rockefeller Foundation; permanent address: Institut Pasteur, Paris 15 (France).

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It is important to remember that the adsorption process is "stopped" by dilution. If bacteria and phage engaged in a reversible union preliminary to the irreversible fixation of the virus particle, then some reversibly held phages would also be counted as being adsorbed at the time of the dilution because *they* could complete whatever reactions are necessary for being registered as adsorbed at some later time without having to encounter another bacterial cell.

The points of curve B of Fig. 1 present the result of a typical adsorption rate measurement at 15° C. The fraction of the input phage adsorbed is plotted as a function of the time between addition of phage to bacteria and dilution. It is seen that the fraction adsorbed is initially proportional to time, and hence we may write

$$dp/dt = r\,(1-p) \qquad (1)$$

where p is the fraction of the phage input adsorbed and r a rate constant.

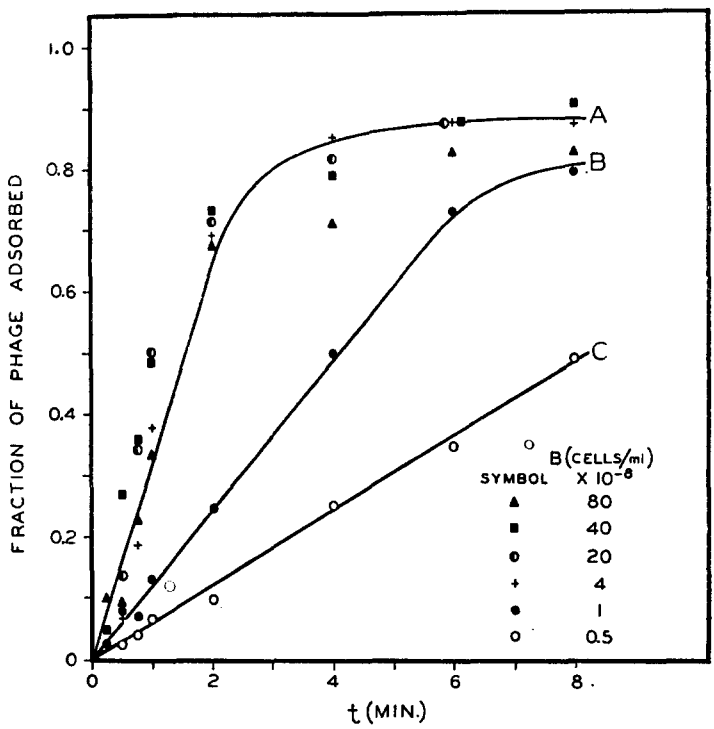

Fig. 1. Rate of adsorption of T4 bacteriophage at various bacterial concentrations, 15° C

COAGULATION EQUATION

SCHLESINGER carried out an analysis of the nature of the rate constant, r, of this relation by postulating that contact between phages and bacteria arises through random collisions of the two bodies. Adsorption may then be treated as diffusion of small particles (the phages) towards spheres of radius a and in concentration B (the bacterial cells) on which the particles are bound irreversibly at a fraction f of all collisions. This

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is precisely the situation described by the VON SMOLUCHOWSKI coagulation equation, from which it may be found that

$$r = 4\pi a D f B \tag{2}$$

where D is the diffusion constant of the phage particles.

As will be seen below, it is necessary to specify more precisely the meaning of the factor f. If f were connected, for instance, with a distribution of the phage population into adsorbable and non-adsorbable classes, then the adsorbable class might be preferentially depleted and a change in f occur during the course of adsorption. Hence, we define f as the fraction of all virus-host cell collisions leading to irreversible union *at the moment when suspensions of phage and bacteria are first mixed.*

One may calculate the value of the ratio r/f from equation (2) by using the most recent values of the diffusion constant of T4 bacteriophage (PUTNAM[5])

$$D = 4.8 \cdot 10^{-6} \text{ cm}^2/\text{min}$$

and letting $a = 8 \cdot 10^{-5}$ cm (this being the radius of a sphere having a surface area equal to that of a cylinder of height 2 μ and width 1 μ). For the bacterial concentration $B = 10^8$ cells/ml employed in the experiment reported as curve B in Fig. 1 it is then found that

$$r/f = 4\pi \cdot 8 \cdot 10^{-5} \cdot 4.8 \cdot 10^{-6} \cdot 10^8 = 0.5 \text{ min}^{-1}$$

But from the initial slope of curve B of Fig. 1 we may find r directly to be

$$r = 0.12 \text{ min}^{-1}$$

from which it follows that f must be of the order of 0.2. In other words, an appreciable fraction of all random collisions between phage and host cell leads to irreversible union of the two.

ADSORPTION RATES AT DIFFERENT BACTERIAL CONCENTRATIONS

The rate of adsorption at different bacterial concentrations B was measured repeating the experimental procedure described above at a number of other host cell densities. The results are presented as the remaining points in Fig. 1, as well as in Table I where the values of r computed from Fig. 1 are reported.

TABLE I

RATE CONSTANTS OF ADSORPTION AT DIFFERENT BACTERIAL CONCENTRATIONS 15° C

Bact. conc. B (cells/ml) $\cdot 10^{-8}$	r (min^{-1})
80	0.3 ± 0.1
40	0.3 ± 0.1
20	0.3 ± 0.1
4	0.3 ± 0.1
1	0.12
0.5	0.06

The fraction of the phage input adsorbed appears to be initially proportional to the time of adsorption at all cell concentrations. At low bacterial concentrations B the rate constant r is proportional to B, as stated by equation (2), but at high B, r becomes independent of B. Hence the model of the adsorption process on the basis of which equation (2) was derived is an adequate description only at low bacterial concentrations and must be amended in some way to include the observations made at high cell densities.

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TEMPERATURE DEPENDENCE OF ADSORPTION

The model referred to above may be probed further by observing the rates of adsorption at different temperatures. For this purpose, a number of experiments were performed, similar to the ones reported in Fig. 1, but keeping the adsorption mixture at temperatures ranging from 5° C to 37° C. The bacterial concentration chosen (10^8 cells/ml) was sufficiently low so that equation (2) retained its validity in each case.

The results of this series of experiments are presented in Fig. 2, where the logarithm of the observed values of r is plotted against the reciprocal of the absolute temperature at which they were measured (curve A). It is seen that lowering of the temperature effects a great reduction of this rate constant.

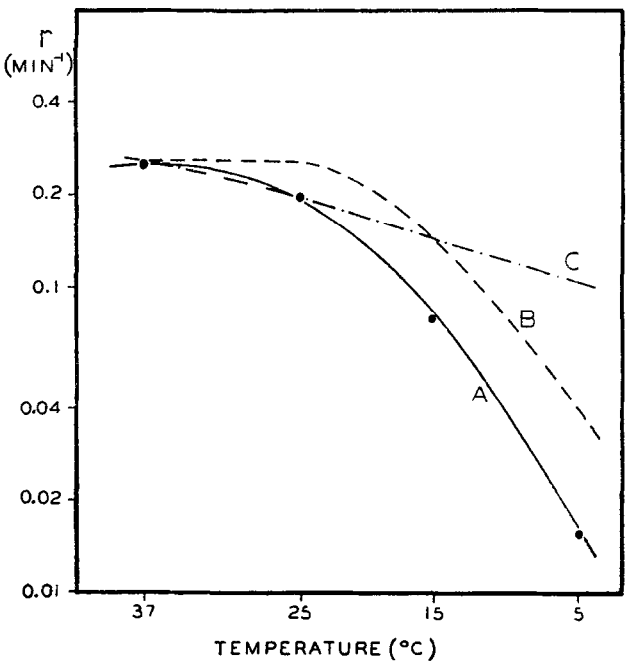

Fig. 2. Rate of adsorption of T4 bacteriophage at various temperatures
Bacterial concentration 10^8 cells/ml
Curve A. Observed rate of adsorption
Curve B. Relative temperature dependence of f (Ordinate arbitrary)
Curve C. Relative temperature dependence of the bacteriophage diffusion constant D (Ordinate arbitrary)

The temperature dependence of r may now be interpreted on the basis of equation (2). Of the parameters appearing in this relation only D, the diffusion constant, and f, the fraction of all collisions leading to irreversible union of virus and host cell, are sensitive to changes in temperature. Of these, the diffusion constant may be expressed in terms of the EINSTEIN equation

$$D = k\,T/6\,\pi\varrho\eta$$

where ϱ is the radius of the virus particle, η the viscosity of the medium in which diffusion takes place, T the absolute temperature and k the BOLTZMANN constant. If it is assumed that η changes in our system as the viscosity of water, relative changes in T/η, and hence in D, over the temperature intervals employed in our measurements may be calculated directly. The results of such calculations are presented as curve C in Fig. 2. Thus curve A of Fig. 2, showing the temperature dependence of r, or by virtue of equation (2) that of the product $f \cdot D$, may now be corrected for changes in D, and the resulting curve B of Fig. 2 then represents *relative* changes in f alone over the temperature range 5° C–37° C. It appears that the fraction of phage-bacterial collisions resulting in irreversible union of the two is independent of temperature between 37° C and 25° C but decreases with temperature between 25° C and 5° C.

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TEMPERATURE DEPENDENCE OF THE MAXIMUM ADSORPTION RATE

In order to form a clearer picture of the nature of the maximum rate of adsorption r_{max} observed at high bacterial concentrations, it was now of interest to investigate the dependence on temperature of this maximum rate. For this purpose we repeated the type of experiment reported in Fig. 1 and Table I at a reduced temperature. In Table II the observed values of the adsorption rate constant r are presented as function of the bacterial concentration B at the temperature of 5° C.

TABLE II

RATE CONSTANTS OF ADSORPTION AT DIFFERENT BACTERIAL CONCENTRATIONS
5° C

Bact. conc. B (cells/ml) $\cdot 10^{-8}$	r (min^{-1})
130	0.085
43	0.079
13	0.080
4.3	0.027
2.2	0.016
0.87	0.008
0.32	0.002

At 5° C, just as was found to be the case at 15° C (*cf.* Table I), there is proportionality of r to B and independence of B at high B. The maximum rate of adsorption at 5° C is, however, significantly lower than that at 15° C, the two being in the ratio

$$\frac{r_{max}\ (5°\ C)}{r_{max}\ (15°\ C)} = \frac{0.08}{0.31} = 0.26$$

Hence the temperature dependence of r_{max} in the interval 5° C to 15° C is quite similar to that of r at a given low bacterial concentration, since from curve A of Fig. 2 it may be seen that for $B = 10^8$ cell/ml

$$r(5°\ C)/r(15°\ C) = 0.20$$

TWO-STEP REACTION

Those of our observations which are not to be expected from simple two-body collision processes may then be summarized:

1. The rate of adsorption reaches a maximum on raising the bacterial concentration, and

2. the fraction of all collisions leading to adsorption decreases with temperature.

An extension of the SCHLESINGER model of bacteriophage adsorption is, therefore, necessary. To account for these findings, it appears that a *second step*, either preceding or following collision, must be included in the model, and three simple alternatives for such an additional step shall be discussed. For each, the starting point is a postulation of a cause why a fraction 1-f of the collisions do not result in adsorption of the phages; the maximum rate of adsorption observed will, in each of the alternatives, be shown to be the rate at which these phages gain another chance of undergoing an irreversible, or "successful", collision.

(i) *Activity-inactivity theory.* If it is assumed that each phage oscillates between one of two states, an "active" state in which collision with a sensitive bacterium leads to certain, irreversible adsorption, and an "inactive" state in which collision leads to rapid separation of phage and sensitive cell without adsorption, then the fraction of collisions leading to adsorption, f, is equal to the fraction of phages in the active state at any instant. At high bacterial concentrations, this fraction of the phages would be adsorbed very rapidly and the remainder, consisting of 1-f of the phage input, would be adsorbed

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at the rate c_a at which the phages make their transition from the inactive to the active state, this rate being independent of the bacterial concentration. Hence $r_{max} = c_a$. If c_d is the rate at which the phages pass to the inactive from the active state, then $f = c_a/(c_a + c_d)$. Under this view, the decrease in f with temperature observed at low B indicates that, at equilibrium, fewer phages are in the active state at the lower temperature.

(ii) *Alternative collision theory.* If it is assumed that the phages do not differ from one another in their adsorbability at any instant but that they may collide with sensitive cells in either a "good" way, which results in certain adsorption, or in a "bad" way, which has no physiological results other than that the phage is held to the bacterium temporarily and reversibly, then the fraction of collisions leading to adsorption is the probability that any collision occurs in the "good" way. Following a "bad" collision, the phage must be freed from the temporary union with the bacterial cell before again being eligible for another try at being adsorbed. At high bacterial concentration, all phages are rapidly brought into their first contact with bacteria. A fraction f is adsorbed at its first collision; the remainder consisting of $1-f$ of the phage input is held in the "bad" way, and may be adsorbed with a rate constant fc_d where c_d is the rate at which the reversible phage-bacterium union is dissolved, this rate being independent of the bacterial concentration. Hence, $r_{max} = f \times c_d$.

(iii) *Surface reaction theory.* If it is, finally, assumed that phage and bacteria enter into a reversible attachment after *each* collision but that subsequent to this attachment the phage may undergo either an irreversible fixation to the bacterium with probability c_a per time unit or free itself from the reversible attachment with probability c_d per time unit, the fraction of the collisions leading to adsorption f may be written $f = c_a/(c_a + c_d)$. At high bacterial concentrations all phages are rapidly brought into the reversible attachment. If bacteria and phage are again separated by dilution shortly after having first been mixed, a fraction f will not free itself from the reversible attachment and will thus be registered as immediately adsorbed. The observed rate of adsorption of the remainder is then c_a, this rate being independent of the bacterial concentration. Hence $r_{max} = c_a$. Under this view, the decrease of f with decreasing temperature may be attributed to a decrease in c_a.

The conceptual difference between the three theories proposed is clearly one of the time at which it is decided whether a given virus-host cell collision will lead to adsorption of the particle or not. Under the *activity-inactivity* theory this decision occurs *prior* to the collision, depending on which of the two classes, "active" or "inactive", the phage particle happens to belong. Under the *alternative collision* hypothesis the decision of whether a collision leads to adsorption takes place *at the instant* of the collision itself, whereas under the *surface reaction* theory this decision occurs some time *after* the collision, while the phage particle is in a state of reversible attachment. If a reversible attachment of the type required for either of the latter two theories could actually be demonstrated, then these two possibilities might be distinguished operationally from each other if some means were found to prolong the length of time of the reversible attachment. For if r_{max} is measured under conditions of decreased c_d, it should show a decline if the *alternative collision* hypothesis were true, whereas it should remain unaltered were the *surface reaction* theory the correct one.

The data reported thus far may now be interpreted in terms of these theories. Under all three alternative views, the fraction adsorbed instantaneously at high bacterial

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concentration is f, an apparent contradiction to the observation that the fraction of the phage input initially adsorbed is proportional to the time (Fig. 1). We must, therefore, place an upper limit on f sufficiently low so that an instantaneously adsorbed fraction f would escape our detection, thus restricting the fraction of successful collisions between this limit and the limit imposed upon it by calculations of the collision frequency. We assess f to be of the order of 0.1 since an instantaneously adsorbed fraction much greater than this figure should not have gone unnoticed and since the calculations of f based on equation (2) should not be in error by very much more than a factor of two.

The approximate order of magnitude of the rate constant c_d can be estimated from r_{max}, the maximum rate of adsorption. It may be readily seen that under each of the three alternative theories, if f is sufficiently small,

$$r_{max} = f\, c_d \tag{3}$$

If we set f equal to one-tenth at 15° C, we find

$$c_d = 0.31/0.1 = 3 \text{ min}^{-1}$$

indicating that the half-life of the phage in the "active" state or in the reversibly attached condition is of the order of 15 seconds.

It is also possible to estimate the temperature dependence of c_d from the observed temperature dependence of r_{max} since according to equation (3)

$$\frac{r_{max}\ (5°\ C)}{r_{max}\ (15°\ C)} = \frac{f\ (5°\ C)\ c_d\ (5°\ C)}{f\ (15°\ C)\ c_d\ (15°\ C)} = 0.26 \tag{4}$$

From the relative values of f at different temperatures shown in curve B of Fig. 2 we find

$$f\ (5°\ C)/f\ (0.15°\ C) = 0.29$$

and hence substituting in equation (4) and solving we find

$$c_d\ (5°\ C)/c_d\ (15°\ C) = 0.9$$

It is evident, therefore, that c_d is quite insensitive to changes in temperature over the interval 5° C to 15° C.

RESISTANT BACTERIA

The possibility was considered whether the reversible reaction between phage and host cell under either the alternative collision or surface reaction theories could occur between virus particles and so-called *resistant* bacterial cells. (A *resistant* bacterial strain originates from a mutant cell which has lost the property of forming an irreversible union with phage particles of types with which preceding generations could still form such union.)

If such resistant bacterial cells can engage phage particles in the same reversible union as postulated for normal, sensitive cells, *i.e.*, that cell and virus separate after each collision with rate c_d, then the presence of resistant cells should decrease the observed adsorption rate of phages to sensitive cells. In a mixture consisting of sensitive

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cells in low concentration, resistant cells in a high concentration B', and phages, there will only be a fraction R of the unadsorbed phages *available for adsorption to sensitive cells at any instant* because of the equilibrium

$$\text{Free phages available for adsorption} \underset{c_d}{\overset{g}{\rightleftharpoons}} \text{Phages held reversibly to resistant cells}$$

where g is the collision frequency of phages with resistant cells. Hence

$$R = c_d/(c_d + g) \tag{5}$$

That is, R should be the ratio of rates of adsorption to identical concentrations of sensitive cells when measured in the presence or absence of a high concentration of resistant cells.

The collision frequency g between the phages and resistant cells in concentration B' may be estimated from equation (2) and from our measurements of the rate of adsorption r of phages to sensitive cells in concentration B, *i.e.*,

$$g = (r/f)\ (B'/B) \tag{6}$$

Substituting for c_d and g from (4) and (6) into (5) we find

$$R = \frac{r_{max}}{r_{max} + r\ B'/B} \tag{7}$$

In experiments carried out at 15° C, the rate of adsorption of T4 phage to sensitive cells in concentration $6 \cdot 10^7$ cells/ml was measured in the absence and presence of resistant (B/4) cells in concentration $2.5 \cdot 10^{10}$ cells/ml. Under these conditions, according to equation (7) and the data of Table I, rates in the ratio

$$R = \frac{0.31 + 0.12\ (2.5 \cdot 10^{10}/10^8)}{0.31} = 0.01$$

should then be expected. No significant difference in the rate of adsorption was found whether the resistant cells were present or not. Hence one may conclude that if a reversible attachment of phage to bacterial cells takes place, the duration of this attachment is much less in the case of resistant cells than in the case of sensitive cells, and must, therefore, depend on specific factors.

DISCUSSION

The postulation of a second step involved in bacteriophage adsorption can thus account, in a satisfactory manner, for the results of measurements of adsorption rates at high cell concentrations and at different temperatures. Although our observations do not permit a decision as to which of the three proposed mechanisms of a second step is the most probable, some recent, important observations of PUCK, GAREN AND CLINE[6] shed new light on this question. These workers found that phages, suspended in a buffer of particular ionic constitution form a union with bacterial cells in this medium, by virtue of which union the phages are thrown down with the bacteria upon low-speed centrifugation. If the pellet of such centrifugation is then resuspended in distilled water,

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almost all of the phages are eluted from the bacterial cells, demonstrating that a reversible attachment had taken place. These findings point to the *alternative collision* or *surface reaction* theories proposed above. Under the former theory one may now envision that in an ionic environment in which reversible adsorption is observed, the rate c_d at which the phages are freed from the "bad" collisions has been greatly reduced. Under the latter theory, one would envision that the ionic environment favoring reversible adsorption has greatly decreased both c_a and c_d, the rates at which phages are either fixed irreversibly or liberated from the reversible union. In either case, the medium which favors elution of the phages from the state of reversible adsorption again restores c_d to its normal value. The *inactivity-activity* theory could, of course, also be reconciled with the findings of PUCK, GAREN AND CLINE, but more complicated postulations would be required.

In experiments with T4 phage, ANDERSON[7] found that no adsorption takes place in phage-bacterial mixtures which are violently stirred in a Waring blender, although agitation ought to *increase* the collision frequency and hence the rate of adsorption. This observation can most easily be interpreted in terms of the *surface-reaction* theory, by postulating that agitation frees the phage rapidly from the reversible union with the bacterial surface, *i.e.*, increases c_d, and thus decreases f, the fraction of collisions leading to irreversible fixation of the phage. (It is to be noted that an increase in c_d would not lead to a reduced rate of adsorption under the *alternative collision* theory).

PUCK, GAREN AND CLINE observed, furthermore, that the rate at which reversible adsorption takes place exhibits a much lower temperature coefficient than irreversible adsorption, in agreement with our interpretation that the temperature dependence of f is responsible for the greatly reduced rate of adsorption at lower temperatures and the consequent independence of c_d of temperature. They also found that reversible adsorption of phages to resistant bacterial cells does not take place, analogous to our own conclusion that c_d, the measure of the duration of reversible attachment, depends on specific factors.

SUMMARY

The rate of adsorption of bacteriophages to host cells increases proportionally to the bacterial concentration B at low B and reaches a maximum rate which is independent of B at high B. The fraction of phage-bacterial collisions leading to irreversible union of these two bodies, estimated to be of the order of 0.1 at 15° C, decreases with temperature.

Three alternative theories involving a second step besides collision in the mechanism of bacteriophage adsorption are proposed and discussed to account for the experimental observations. One theory envisions the phage particles as oscillating between an "active" and an "inactive" state. The other two theories involve the concept of a reversible attachment of virus to host cell. The half-life of the "active" state or of the reversible attachment is of the order of 15 seconds at 15° C.

RÉSUMÉ

La vitesse d'adsorption des bactériophages sur les bactéries hôtes est tout d'abord proportionelle à la concentration des bactéries lorsque cette concentration est faible, puis elle devient indépendante de celle-ci à fortes concentrations et atteint donc un maximum.

La fraction des collisions phage-bactérie menant à une union irréversible des deux particules diminue avec la température. Elle est estimée être de l'ordre de 0.1 à 15° C.

Pour expliquer les observations expérimentales trois théories alternatives sont proposées et discutées. Toutes trois font intervenir en plus des collisions un second phénomène lors de l'adsorption des phages. La première considère que les phages oscillent entre un état "actif" et un état "inactif". Les deux autres mettent en jeu le concept d'une liaison réversible du virus sur la cellule hôte. La "demi-vie" de l'état actif ou de la liaison réversible est de l'ordre de 15 secondes à 15° C.

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ZUSAMMENFASSUNG

Die Absorptionsgeschwindigkeit von Bakteriophagen an Wirtszellen steigt proportional mit der Bakterienkonzentration B solange diese niedrig ist und erreicht bei hoher Konzentration ein von B unabhängiges Maximum. Der Bruchteil der Anzahl der Berührungen zwischen Phagen und Bakterien, welche zu irreversibeler Bindung führen, wird bei 15° C auf 0.1 geschätzt; er nimmt mit der Temperatur ab.

Drei Phagen-Absorptions-Mechanismen, welche ausser der notwendigen Berührung zwischen Virus und Wirtszelle noch eine zweite Stufe umfassen, werden erörtert um den experimentellen Beobachtungen Rechnung zu tragen. Eine der Theorien setzt eine Oszillation der Phagen-Teilchen zwischen einem "aktiven" und einem "inaktiven" Zustand voraus. Die zwei weiteren Theorien beruhen auf dem Gedanken einer reversibelen Bindung zwischen Virus und Wirtszelle. Die Halbwertszeit des "aktiven" Zustandes oder der reversibelen Bindung ist von der Grössenordnung 15 Sekunden bei 15° C.

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