# THE FIRST TWO STEPS OF THE INVASION OF HOST CELLS BY BACTERIAL VIRUSES.* II

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In an earlier paper (1) experiments with the *Escherichia coli*-bacteriophage system were described elucidating certain aspects of the primary attachment of a virus to its specific bacterial host. It was shown that the initial interaction of virus and host consists of an extremely rapid, reversible step whose rate can be completely controlled by the salt constituents of the medium. Many of the characteristics of this reaction were found to be duplicated in the attachment of bacteriophage to an inorganic ion-exchange surface, like glass. To explain this reaction, it was proposed that when a virus and host cell are placed in a solution of an appropriate ionic composition, ions are first taken up by specific groupings on the two surfaces to form complementary geometrical and electrostatic patterns that permit rapid union of the two bodies. It was also shown that the specific resistance of a given cell mutant (*E. coli* B/1,5) to invasion by the T1 virus is due to failure of this reversible attachment to occur.

Experiments with this and other virus systems (2, 3) have demonstrated the importance in virus reproduction of enzymatic steps which occur very early after infection has taken place. The studies herewith reported were undertaken to unravel the roles of enzymatic transformations and other reactions in the infective process. These studies demonstrate that for the bacterial viruses at least, irreversible reaction partaking of the characteristics of enzymatic transformation occurs as a second step after the initial, reversible binding has taken place. A summary of five different types of experiment which serve to separate these two earliest phases of the virus life cycle is presented here.

## *Methods and Materials*

The procedures employed have already been described (1). Suspensions of cells and virus were mixed in media of composition indicated, maintained in a thermostated water bath. The extent of virus-cell attachment was measured by centrifuging such suspensions and determining the virus content of the supernatant, which represents the virus that had not

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become adsorbed onto cells. Unless otherwise noted, all solutions contained $5 \times 10^{-4}$ M phosphate buffer at pH 7. The ionic requirements for attainment of the maximal theoretical rate of cell attachment for the two viruses employed (1) are as follows: for T1: (0.5–1) $\times 10^{-3}$ M $Ca^{++}$, $Mg^{++}$, $Ba^{++}$, or $Mn^{++}$; or $10^{-2}$ M $Na^{+}$ or other univalent cations. For T2: 0.1 M $Na^{+}$ or $K^{+}$.

## EXPERIMENTAL RESULTS

*1. Temperature Dependence of the Reversible and Irreversible Steps.*—If the initial binding of virus to cell is primarily an electrostatic interaction, its rate will be relatively independent of temperature, in contrast to the rates of subsequent enzymatic steps which should decrease sharply as the temperature is lowered. Thus, almost equally rapid attachment rates should be expected at 37.0°C. and 2.0°C., but only at the latter temperature should a significant fraction of the virus be readily eluted from the cells. Such behavior was observed for the interaction of T1 and *E. coli* B in synthetic media of optimal ionic composition ($10^{-3}$ M $CaCl_2$ or $10^{-2}$ M NaCl), as shown in Table I.

In $10^{-3}$ M $CaCl_2$, the rate constant for virus attachment, defined as $k$ in the equation

$$-\frac{dV}{dt} = kVC$$

(where $V$ is the concentration of virus remaining free at times, $t$, in presence of a host cell concentration, $C$,) has the following values:—

| *Temp.* | $k$ |
|---|---|
| °C. | cm.$^3$ min.$^{-1}$ |
| 37 | $2.7 \times 10^{-9}$ |
| 2.0 | $1.3 \times 10^{-9}$ |

Most enzymatic reactions suffer a ten- to fortyfold reduction in rate over this temperature range. A reaction in which every collision is effective is limited only by the rate of molecular diffusion, which would be altered significantly only by changes in the viscosity of the medium. The viscosity of a dilute salt solution like $10^{-3}$ M $CaCl_2$ should closely parallel that of water, which increases by a factor of 2.4 when the temperature is lowered from 37°C. to 2.0°C. (4). Within experimental error, this factor accounts for the 2.1-fold decrease observed in the specific velocity constant for the attachment reaction.

*2. Effect of Salt Concentration.*—A similar separation of the first reversibel step from the second irreversible one at 37°C. is possible in the case of T2 virus through proper control of the salt concentration in the medium. Thus, T2 attachment to *E. coli* B is almost completely irreversible at 37°C. if the optimum concentration of salt, 0.1 M NaCl, is employed. Lower concentrations produce an attachment which is not only slower, but to a large extent reversible. A typical set of data is presented in Table II.

These results suggest that ions are required for both the first and second steps of the virus attachment. The slowness of the first step at low salt concentrations can be compensated for by increasing the concentration of the bacterial cells, but the enzymic step which occurs on or within each infected

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TABLE I

*Demonstration That in an Optimal Ionic Medium, Virus Attachment Is Almost Equally Rapid at 37°C. and 3°C., and That Only at the Lower Temperature Is the Process Reversible*

T1 bacteriophage ($5 \times 10^4$/cm.$^3$) and *E. coli* B ($2 \times 10^8$/cm.$^3$) were mixed in tubes containing salt solutions allowing rapid attachment (either $10^{-3}$ M $CaCl_2$ or $10^{-2}$ M NaCl) and kept for 10 minutes at the temperatures indicated. These are called the attachment tubes. Then samples from each "attachment tube" were removed, diluted 1:10, and maintained for 15 minutes in elution tubes containing nutrient broth plus an excess of NaCl, (0.1 M) at 0°C., a medium which inhibits attachment and promotes elution of this particular virus (1). Both the original reaction tubes and these elution tubes were then rapidly centrifuged at 0°C., and the virus remaining unattached to cells was determined by titration of the supernatants.

| Attachment medium | Reaction temperature | Per cent of virus becoming attached to cells after 10 mins. in adsorption tube | Per cent of the attached virus re-eluted by dilution in medium containing excess NaCl |
|---|---|---|---|
| | | *per cent* | *per cent* |
| $10^{-3}$ M $CaCl_2$ | 37°C. | 98 | <5 |
| | 3°C. | 88 | 43 |
| $10^{-2}$ M NaCl | 37°C. | 96 | 10 |
| | 3°C. | 82 | 70 |

TABLE II

*Demonstration That the Attachment of T2 Virus to Its Host at 37°C. Is Irreversible in 0.1 M NaCl, but Reversible at Lower Salt Concentrations*

T2 virus ($4 \times 10^6$/cm.$^3$) was mixed with an excess of cells ($3 \times 10^9$/cm.$^3$) at 37°C. for 3 to 5 minutes in the salt solutions indicated, after which time at least 90 per cent of the virus had become attached to cells. The suspensions were centrifuged, the cells separated, and then resuspended in distilled water at 0°C., an effective eluting medium (1) for this virus. After an equilibration period of 5 minutes, the cells were again separated by centrifugation, resuspended in fresh elution medium, and again centrifuged. The total amount of virus liberated from the cells into the supernatant in these two elution steps is indicated in the table.

| Concentration of NaCl in the original adsorption tube at 37°C. | Elution medium | Per cent of adsorbed virus eluted |
|---|---|---|
| | | *per cent* |
| 0.10 M | $H_2O$ at 0°C. | 16.2 |
| 0.04 M | " " " | 51.0 |
| 0.02 M | " " " | 73.5 |

cell remains inhibited at the low salt concentration. This makes possible separation of the two reactions.

These experiments raise the question as to which step of virus attachment is

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involved in the killing of the host cell. Such killing has been shown to result from the attachment of a virus, even when the virus has been previously inactivated by treatment with ultraviolet light (5). As might have been expected on the basis of the general picture here developed, the reversible attachment of T2 to *E. coli* B which occurs in 0.02 M NaCl at 37° does not kill the cell, whereas attachment in 0.10 M NaCl at the same temperature does (Table III). The conclusion may be drawn that the initial, reversible interaction leaves both virus and host essentially intact, but is rapidly followed by profound alteration of some vital part of the cell by the virus.

TABLE III

*Demonstration That the Reversible Attachment of T2 to E. coli B in 0.02 M NaCl at 37°C. Does Not Kill the Cell*

T2 virus (approximately $5 \times 10^8$ cc.) and *E. coli* B cells ($3 \times 10^8$/cc.) were added to the media indicated and kept for 15 minutes at 37°C. The per cent of virus which became attached to cells was determined by centrifugation of aliquots from each tube and titration of the free virus in the supernatant. Then samples from each tube were titred for total viable cell content by a plating on nutrient agar *without* added NaCl, a medium which permits growth of the cells, but not of the T2 virus.

| Medium | Per cent of virus attached to cells after 15 mins. at 37°C. | Per cent of cells killed as result of virus attachment |
|---|---|---|
| | *per cent* | *per cent* |
| (*a*) 0.02 M NaCl (reversible attachment) | 91 | 0 |
| (*b*) 0.10 M NaCl (irreversible attachment) | 88 | 98 |

In the absence of virus, the cells remain viable in both media.

*3. Inhibition of the Second Reaction by Specific Chemical Agents.*—A search for inhibitory chemical agents led to the finding that $Zn^{++}$ ion specifically blocks the second reaction in the invasion of *E. coli* B by T1 virus (6). An important feature of this action of Zn is that it is virus-specific,—for example, it does not prevent the irreversible step in the adsorption of T2 virus, as it does with T1 (7).

$Zn^{++}$ ion promotes the primary attachment of T1 to cells of *E. coli* B just as effectively as does $Ca^{++}$ or $Mg^{++}$. In the presence of $(0.5-1.0) \times 10^{-3}$ M $ZnCl_2$ or $Zn(NO_3)_2$ and in the absence of any other salt, the specific velocity constant for attachment is approximately $1 \times 10^{-9}$ cm.$^3$ min.$^{-1}$, a value in the neighborhood of 100 per cent collision efficiency. $Zn^{++}$ also promotes the reversible attachment of T1 virus to glass, a reaction which has many features in common with the first step of cell invasion (1).

Since $Zn^{++}$ fails to bring about the second step, the system of T1 phage and host cell in the presence of Zn displays only reversible attachment. Thus, if a T1-cell mixture containing $10^{-3}$ M $Zn^{++}$ is centrifuged and the supernatant

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titred, most of the virus is found to be attached to cells. However, if the mixture is diluted 1:10 in nutrient broth at 0°C. before centrifugation, most of the virus is liberated from the cells and recovered in active form (Table IV).

$Zn^{++}$ not only fails by itself to promote the subsequent steps of T1 invasion but also prevents $Ca^{++}$ or $Mg^{++}$ from doing so. If T1 virus and *E. coli* B are mixed in phosphate buffer containing the optimal concentration of $Ca^{++}$ for irreversible attachment ($5 \times 10^{-4}$ M) plus an equimolar concentration of $ZnCl_2$, only the first, reversible step occurs. The presence of the $Zn^{++}$ protects the cells against any permanent damage.[1] The virus can still attach to the outside of the

TABLE IV

*Demonstration That $10^{-3}$ M $Zn^{++}$ Promotes Only the Reversible Step of Virus-Cell Attachment*

T1 virus ($3 \times 10^4$/cm.³) and *E. coli* B ($2 \times 10^8$/cm.³) were mixed and maintained for 5 minutes at 37°C. in the presence of (*a*) $10^{-3}$ M $Zn(NO_3)_2$ and (*b*) $10^{-3}$ M $CaCl_2$. Rapid cell attachment took place in each tube, but only that of the Zn tube was reversed by a 1:10 dilution in nutrient broth at 0°C. The pH of both solutions was adjusted to the same value, 5.8.

| Reaction medium (37°C.) | Per cent of virus attached to cells | Per cent of attached virus which was re-eluted by dilution in broth at 0°C. |
|---|---|---|
| | *per cent* | *per cent* |
| (*a*) $10^{-3}$ M $Zn(NO_3)_2$ + $10^{-4}$ M phosphate buffer | 70 | 71 |
| (*b*) $10^{-3}$ M $CaCl_2$ + $10^{-4}$ M phosphate buffer | 95 | $<7$ |

cell, but cannot secure a foothold in the cellular metabolic machinery. Data illustrative of this protective action of zinc are presented in Fig. 1.

In order to test whether this inhibition of the enzymic phase of T1 invasion is due to reaction of the Zn with the virus or the cell, the following experiment was designed: First T1 virus was incubated for 10 minutes at 37°C. in an inhibitory concentration of $Zn^{++}$ ($5 \times 10^{-4}$ M). A small aliquot of this mixture was then removed and added to a large volume of cells in a medium containing optimal $Ca^{++}$ concentration, so that in the resulting virus-cell suspension the concentration of Zn was negligibly small. Under these conditions, virus and cell united irreversibly at an over-all rate identical with that of experiments in which Zn had not been employed (curve B, Fig. 1). The converse procedure, however, had a different result: If the cells alone were first exposed to $5 \times 10^{-4}$ M $Zn^{++}$, and then a small aliquot quickly added to a large volume of virus in a medium promoting rapid attachment, the rate of the irreversible union of virus to these cells was inhibited to practically the same extent as in the A

[1] If an excess of zinc is employed, toxic action on the cells may result. With Zn concentrations of $5 \times 10^{-4}$ M, however, virtually complete protection against the virus is secured with little reduction in the number of viable cells.

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curve of Fig. 1, in which Zn was actually present in the attachment medium. Since cells alone which have been exposed to Zn suffer marked depression in the rate of irreversible interaction with T1 virus, whereas virus alone which has been exposed to Zn attaches to cells in completely normal fashion, the

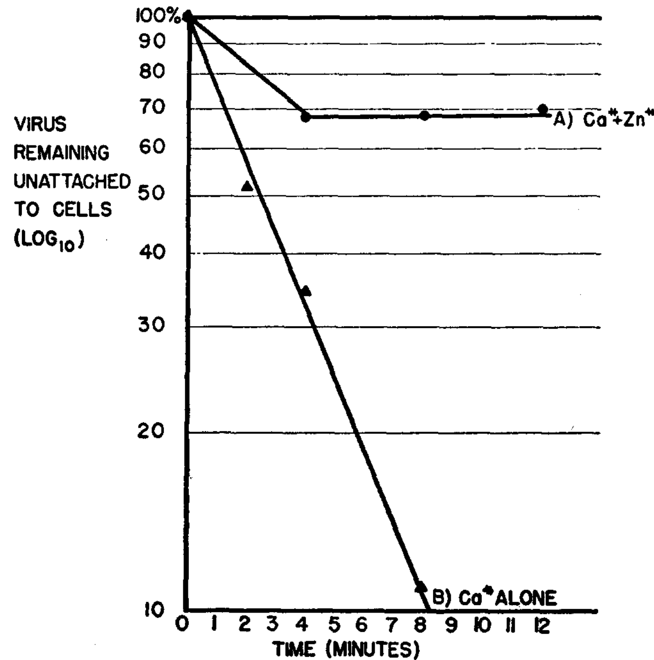


**FIG. 1.** The inhibition by $Zn^{++}$ of the action of $Ca^{++}$ in bringing about the irreversible step of T1 invasion of *E. coli* B. The plotted points represent all the virus which had not become ***irreversibly*** attached to host cells.

Virus and cells were mixed at 37°C. in (A) $7.5 \times 10^{-4}$ M $CaCl_2$ + $1 \times 10^{-3}$ $ZnCl_2$ and (B) $7.5 \times 10^{-4}$ M $CaCl_2$ alone. The T1 concentration was $5 \times 10^4$/cm.$^3$, and the cell concentration $1.4 \times 10^8$/cm.$^3$. At the times indicated, aliquots were removed and diluted in nutrient broth at 0°C. to allow dissociation of any reversibly bound virus. The dilution tubes were centrifuged and the free virus content determined by titration of the supernatant.

presumption is strong that the component responsible for the irreversible phase of the binding of T1 to its host cell, is located on the cell, rather than the virus.

Tracer experiments with the radioactive isotope, $Zn^{65}$, made possible the demonstration that, on the average, each cell of *E. coli* B must take up $4 \times 10^7$ atoms of Zn in order to block completely the irreversible step of T1 invasion. Moreover, the existence of a competition for the same cellular sites between $Zn^{++}$ and the ions like $Ca^{++}$ or $Mg^{++}$ which promote the normal sequence of reactions in an infected cell, was also demonstrable by means of labelled-Zn

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experiments. Fig. 2 indicates the extent of Zn uptake by cells of *E. coli* B in the presence and absence of $Mg^{++}$ respectively. A detailed account of these experiments which also shed light on differences in the invasion of the same host by different viruses will be presented elsewhere (7).

4. *Action of Ultraviolet Radiation.*—On the basis of the theoretical formulation presented, it appeared likely that ultraviolet irradiation of the *E. coli* cells alone should inhibit selectively the second step of virus invasion, leaving

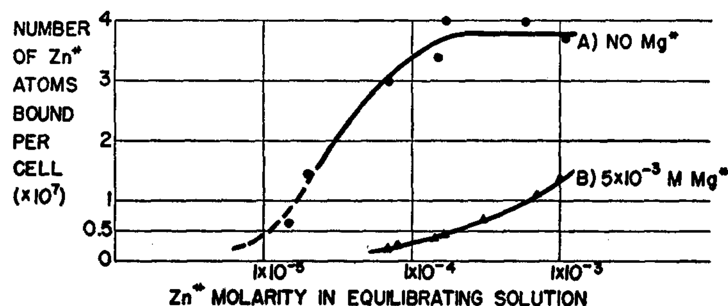


FIG. 2. Uptake of zinc by cells of *E. coli* B, measured with the radioisotope $Zn^{65}$.

A curve: Young cells of *E. coli* B, grown in nutrient broth with aeration, were washed and resuspended ($2 \times 10^9$ cells/cm.$^3$) in solutions of varying $Zn(NO_3)_2$ concentrations buffered at pH 6 with phosphate buffer. Each solution contained a tracer amount of radioactive $Zn^{65}$. The cells were equilibrated for 4 minutes at 37°C., then centrifuged, and washed in distilled water. Test revealed that the $Zn^{++}$ taken up by the cells was not eluted in the wash medium. The cells were again centrifuged, separated from the supernatant, and their $Zn^{65}$ activity determined with a thin-window (1.6 mg./cm.$^2$) Geiger tube. The activity in the supernatant from the first centrifugation was also counted. The sum of the activities in the cells and the original supernatant added up to the total initial activity within ± 4 per cent. The abscissae of the curve represent the $Zn^{++}$ concentrations in solution after the equilibration period.

The B curve was determined in an identical manner, except that $5 \times 10^{-3}$ M $MgCl_2$ was also present in each equilibration tube.

the first step relatively unaffected. The basis for this prediction lay in the fact that most of the groups responsible for ionic interaction in biological systems,—carboxyl, amino, substituted amino, hydroxyl, sulfhydryl, and substituted phosphoric acid, have low ultraviolet absorption coefficients in the neighborhood of 2500 A. Furthermore, the ability of these groups to undergo ion-exchange reactions is not dependent on the structural integrity of the rest of the molecule to which they may be attached, and so exposure to ultraviolet should not appreciably affect the ionic interactions characteristic of these groupings. Hence, the initial attachment reaction should be resistant to the action of such radiation. On the other hand, enzymic transformations which involve the opening of covalent bonds may be expected to depend on the es-

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sential integrity of a large protein molecule containing many cyclic aromatic groupings. The high ultraviolet absorption coefficient of these components should render the enzymatic function of the molecules containing them susceptible to inactivation by ultraviolet light.

Experiment confirmed this expectation. Cells of *E. coli* B exposed to a large dose of ultraviolet irradiation were found to bind T1 virus with exactly the same high attachment velocity as normal cells. However, the attachment of T1 to such irradiated cells in $10^{-3}$ M $CaCl_2$ at 37°C. is completely reversible. When the infected, irradiated cells are resuspended in a medium in which one

TABLE V

*Demonstration That Ultraviolet Irradiation of Host Cells Specifically Inhibits the Irreversible Step of Virus-Host Attachment*

Cells of *E. coli* B, suspended in phosphate buffer, were irradiated for 1 hour, at a distance of 1 meter from a 30 watt General Electric low pressure ultraviolet lamp, which emits most of its energy in the neighborhood of 2537 A. The fraction of viable survivors was less than 0.01 per cent. T1 virus was added to such a cell suspension in $10^{-3}$ M $CaCl_2$ and the mixture maintained for 10 minutes in a 37°C. bath. An aliquot was removed from the tube and diluted 1:10 in an elution tube containing nutrient broth + 0.1 M added NaCl. Both the original tube and the dilution tubes were centrifuged and the virus content of their supernatants determined.

| Cells | Per cent of virus attached to cells after 10 mins. in $10^{-3}$ M $CaCl_2$ at 37°C. | Per cent of attached virus eluted by nutrient broth + 0.1 M NaCl |
|---|---|---|
| | *per cent* | *per cent* |
| Ultraviolet-irradiated | 84 | 84 |
| Normal | 99.5 | 3 |

would expect elution, almost all of the virus is recovered in free form. Experimental details are presented in Table V. The reversibility of this reaction in such irradiated cells makes this system particularly suitable for measurement of the equilibrium constants of the initial attachment (8).

*5. The Isolation of Specifically Resistant Cell Mutants.*—Since two separate steps are necessary in order for a virus irreversibly to infect a cell, two different kinds of specific cellular resistance to a virus might be expected, depending on which step is blocked. Earlier experiments (1) had demonstrated that the *E. coli* mutant B/1,5 is immune to the action of T1 phage because of failure of the first, reversible reaction to occur. In the present study, it was found that *E. coli* B/1, another mutant completely resistant to T1 virus but susceptible to other phages of the T series, owes its specific T1 immunity to a block of the second reaction. In $10^{-3}$ M $MgCl_2$ this mutant binds T1 virus with the maximum rate. However, the bound virus is unable to carry forward the subsequent irreversible step of its life cycle, and the attachment obtained is completely reversible. Table VI illustrates a typical experiment comparing the behavior of T1 toward the three *E. coli* mutants, B, B/1, and B/1,5.

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Like that of B/1,5, the specific resistance of the *coli* mutant, B/2 (in this case to T2 bacteriophage) resides in failure of the first attachment to be established. The B/2 cell binds T1 virus with maximum velocity and promotes its subsequent normal multiplication, but does not bind T2 under any conditions tested. Studies on other virus-cell combinations are in progress, with the hope of elucidating biochemical and genetic factors responsible for these specific metabolic blocks.

TABLE VI

*Demonstration That T1 Virus Undergoes both Reversible and Irreversible Interaction with E. coli B; Only Reversible Attachment with E. coli B/1; and Neither Reaction with B/1, 5*

T1 virus ($5 \times 10^3$/cm.$^3$) and cells ($2 \times 10^8$/cm.$^3$) were mixed in $10^{-3}$ M $Mg^{++}$ at 37°C. for 9 minutes, then centrifuged directly, and the supernatants titrated. Wherever attachment had taken place, its reversibility was measured by resuspending an aliquot of the total suspension in broth + an excess of NaCl, and titrating the supernatant after a second centrifugation.

| Nature of the cells employed | Per cent of virus attached to cells | Per cent of attached virus eluted by treatment with nutrient broth + 0.1 M NaCl at 0°C. |
|---|---|---|
| | *per cent* | *per cent* |
| *E. coli* B | 97 | <6 |
| *E. coli* B/1 | 82 | 100 |
| *E. coli* B/1,5 | <4 | |

TABLE VII

*Experiment Indicating That the Tryptophane-Requiring Mutant of T4 Bacteriophage Utilizes the Tryptophane for the Initial Attachment Step in Host Cell Invasion*

Cells of *E. coli* B ($2 \times 10^8$/cm.$^3$) and the tryptophane-deficient mutant of T4 ($5 \times 10^4$/cm.$^3$) were mixed at 37°C. in 2 tubes containing 0.10 M NaCl + 0.001 M $MgSO_4$. One tube also contained $20\gamma$/cm.$^3$ of *l*-tryptophane. At the end of 16 minutes, both tubes were plunged into an ice bath. Aliquots from each tube were removed and diluted 1:10 in nutrient broth at 0°C., in order to elute any reversibly adsorbed virus. Then both the original tubes and the dilution tubes were centrifuged at 0°C. and aliquots of all supernatants titrated for their virus content. If reversible attachment had occurred in the tube without tryptophane, the supernatant of the original attachment tube should have a low virus titre, while that of the dilution tube should be high. The absence of any such effect indicates that not even reversible attachment occurs. In the tube containing tryptophane both steps take place.

| Tryptophane concentration in attachment tube | Total virus concentrations | Virus titre in supernatant of original attachment tube | Virus titre in supernatant of dilution tube |
|---|---|---|---|
| 0 | $5.3 \times 10^4$ | $4.7 \times 10^4$ | $4.9 \times 10^4$ |
| $20\gamma$/cm.$^3$ | $6.5 \times 10^4$ | $0.3 \times 10^4$ | $0.2 \times 10^4$ |

The role of tryptophane in cell attachment of the tryptophane-deficient T4 virus is of interest in this connection. This particular bacteriophage cannot

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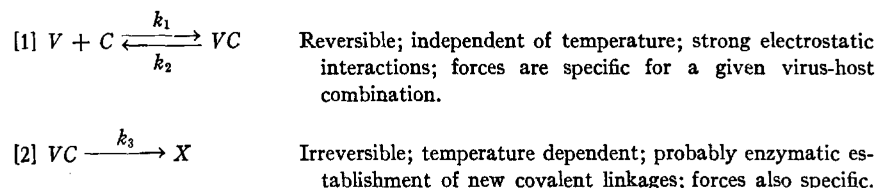
invade host cells in the absence of this specific organic cofactor (9). In view of the metabolic role of tryptophane in various biological systems, it might have been expected that this molecule would be required for the second, irreversible step, presumably enzymic in character. However, earlier studies (1) had demonstrated that this particular bacteriophage requires tryptophane in order to attach to a glass filter, a substrate which was found to be an accurate model in many respects for the role of the cell in the *first* attachment reaction. On this basis, it would be expected that the tryptophane requirement should involve the initial attachment of the bacteriophage to the cell. Such was demonstrated to be the case. A typical experimental protocol reproduced in Table VII illustrates this action.

## DISCUSSION

The reaction scheme proposed (1) for the sequence of events involved in the infection of a host cell, $C$, by a bacterial virus, $V$, may be represented as follows:—



[1] $V + C \underset{k_2}{\overset{k_1}{\rightleftharpoons}} VC$ Reversible; independent of temperature; strong electrostatic interactions; forces are specific for a given virus-host combination.

[2] $VC \xrightarrow{k_3} X$ Irreversible; temperature dependent; probably enzymatic establishment of new covalent linkages; forces also specific.

The symbol $X$ indicates the first metabolic product of the infected unit whose formation is irreversible under the conditions of these experiments. Other two-step schemes had previously been postulated for the attachment of bacteriophage to host cells (2, 9), but these pictured the first step as a nonspecific reaction with weak binding forces.

It is not necessary to visualize the reactions [1] and [2] as occurring at different points in space. The site of the initial virus attachment might be located on the same enzyme molecule which carries forward reaction [2] particularly since under ordinary conditions, reaction [2] follows the initial attachment very swiftly. Thus, although the initial step is not enzymic in the usual sense of the word, which implies establishment of new covalent bonds, it is rapidly followed by such a transformation, and it seems possible that both steps involve the same, or at least adjacent, molecules in the cell surface. This formulation is consistent with the dynamics of other enzyme actions and would appear to reconcile some of the conflicting views which have been proposed concerning the enzymatic or non-enzymatic nature of virus-host attachment (3).

The existence of these two separate reactions affords a prospect for obtaining blocking agents which can operate at two different points in protecting a cell against a virus invader. Thus, $Zn^{++}$ acts as a specific prophylactic agent

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for the T1–*E. coli* B system by preventing reaction [2]. Other experiments will shortly be described in which reaction [1] can be prevented by other substances. These principles might perhaps furnish a basis for prophylaxis against some mammalian viruses.

Elucidation of the detailed nature of the binding forces responsible for the initial attachment is still required. That electrostatic forces due to ionic charges play a significant role cannot be doubted (1); the extent to which these forces are due directly to specific chemical groupings on the surfaces of the two bodies, and to the ionic double layer which forms about such macromolecular structures as a result of the presence of primary polar groups, remains to be determined. The concept that some kinds of biological specificity may reside in the distribution of ionic groupings producing strong electrostatic forces, as well as in weaker van der Waal's and multipolar interactions, is being tested experimentally in studies now in progress.

The fact that the rate of the primary attachment reaction has no significant temperature coefficient between 37°C. and 1.0°C. supports the conclusion reached earlier (10) that the initial attachment proceeds with a collision efficiency approaching 100 per cent. If almost every random collision of a virus particle with a host bacterium can result in reaction, the heat of activation of the system must be very low, and the temperature coefficient of the reaction velocity constant, $\frac{d \ln k}{dT} = \frac{\Delta H_{\text{activation}}}{RT^2}$ should be correspondingly small, as was found to be the case. The fact that the velocity of this first step is nearly independent of temperature was not observed by previous investigators because the experimental techniques used in most of the studies hitherto reported (10) provided only the over-all rate of the first two reactions combined.

The present experiments demonstrate that, although temperature change has little effect on reaction [1], it strongly influences reaction [2]. On the assumption that the reversal of the first reaction is not strongly influenced by temperature, an assumption which has been confirmed experimentally (8), the heat of activation of the enzymic reaction [2] can be calculated from data on the over-all temperature coefficient for the *irreversible* attachment of T1 virus to its host. The value of $\Delta H_{\text{activation}}$ for this irreversible step, so computed from the data of Puck, Garen, and Cline (1), is 18,000 cal./mole, a value in keeping with typical activation energies for enzymic reactions.

The question arises as to whether the ion-binding groups concerned in both the first and second steps of virus invasion are located on the surface of the virus, the cell, or both.

The following facts are pertinent: (*a*) $Zn^{++}$ like $Ca^{++}$ and $Mg^{++}$ (and, in higher concentrations, $Na^{+}$) promotes the first cell attachment reaction of T1; it likewise permits virus attachment to ion-exchange resins; it can also enter into a specific reaction with pure T1 virus which prevents the inactivation

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otherwise occurring in solutions of low ionic strength (11). Hence, it may be concluded that metallic cations can enter into definite chemical combination with the virus molecule and that this ion binding is necessary for cell attachment. (*b*) Zn, however, blocks the second enzymic phase of T1 virus invasion; this action is a result of the binding of Zn to specific sites on the cell. Moreover, this binding of Zn to the cell is a competitive reaction with $Ca^{++}$, $Mg^{++}$, and $Na^{+}$, which promote the normal enzyme operation. The presumption is, therefore, that the second step of virus invasion requires cationic binding to *cellular* chemical groupings. (*c*) The fact that low concentrations of the necessary cations promote the initial attachment of T2 virus to cells at 37°C., but that higher concentrations are required for the progress of the second reaction, is in agreement with these conclusions.

There is some evidence that proteins of nutrient broth may also affect these first two reactions, since at low temperatures anomalies are encountered in the rate of attachment of some viruses when nutrient broth is employed. These effects are receiving further study.

## SUMMARY

At 37°C., the attachment of T1 virus to its host cell in solution containing $10^{-3}$ M $CaCl_2$ or $10^{-2}$ M NaCl is extremely rapid (in the neighborhood of 100 per cent collision efficiency) and irreversible. At 1°C., the attachment rate is almost equally rapid but largely reversible.

If a suboptimal concentration of the necessary ions is employed when T2 virus attaches to host cells, the resulting binding is largely reversible, even at 37°C.

Reversible T2 attachment to host cells leaves the cell undamaged and capable of normal reproduction. Irreversible attachment results in death of the cell.

$Zn^{++}$ exercises a specific inhibitory action on the invasion of *E. coli* B by T1 virus. The virus can still attach to the host cell at a rate closely approximating the maximum value, but the reaction remains reversible and the cell is protected against permanent damage.

The protective action of the Zn against T1 invasion is exerted through an action on the cell, rather than on the virus.

Studies of the uptake of radioactive $Zn^{65}$ show that cells become completely immune to T1 invasion when, on the average, $4 \times 10^7$ atoms of Zn have been taken up by each cell.

Cells killed by ultraviolet irradiation still bind T1 at the maximum rate, but the reaction is reversible even when taking place at 37°C., in optimum salt concentration.

The tryptophane-deficient mutant of T4 bacteriophage requires its specific cofactor for the initial step of attachment to the host cell.

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These experiments support the picture previously developed that virus invasion of host cells consists in an initial, reversible attachment whose properties are those to be expected from the operation of electrostatic binding forces. The step is followed by an enzymatic transformation which is irreversible, strongly temperature-dependent, and in the case of T1 virus, susceptible to inactivation by ultraviolet radiation.

The resistance of mutant cells to specific bacteriophages is of two types, depending on whether the first or second of these steps is blocked.

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