STUDIES ON ACTIVATION OF T4 BACTERIOPHAGE BY COFACTOR

I. THE DEGREE OF ACTIVITY

by

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INTRODUCTION§

It was discovered by T. F. ANDERSON[1] in 1945 that some strains of bacteriophage showed a much lower plaque count when plated on synthetic medium agar than when similarly plated on nutrient broth agar. Addition of certain organic compounds to the phage-bacteria mixture could bring the plaque count on minimum medium agar up to that obtainable on the complete medium. ANDERSON was able to demonstrate that the presence of the active compounds was required for the *adsorption* of these bacteriophages to the host cells, and he designated the compounds *cofactors*.

Of the compounds tested, L-tryptophan was the one which in the lowest concentration could induce full plaque counts on minimum medium. Other amino acids, such as phenylalanine and tyrosine, and some analogues of tryptophan[3], although less effective, were also found to act as cofactors.

More detailed studies of the role of cofactors have so far been confined to the action of L-tryptophan. The adsorbability of the phage depends on such factors as the tryptophan concentration, the composition of the medium, and the temperature. Cofactor action appears, furthermore, to be reversible since the phages revert to their initially inactive condition upon dilution of the tryptophan[4].

The cofactor requirement is a heritable property of the bacteriophage[5, 6]. Mutants with different quantitative requirements have been found. Some may need cofactor only at low temperature but can dispense with it at 37° C[7]. Other mutants differ by their qualitative requirements. Certain strains, for instance, need calcium ions in addition to the presence of tryptophan for adsorption to their hosts. Finally, some mutants which are activable by tryptophan are specifically inhibited by indole[6].

The discovery of cofactors for adsorption of bacteriophages on bacteria has brought the hope that a study of the mode of action of these rather simple molecules may give an insight into the mechanism by which virus becomes attached to host cell. The following investigations, in which many of the methods used were those already employed

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§ The methods and terminology of recent bacteriophage research are described in a review by DELBRÜCK[2] (1946).

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by ANDERSON, and by HERSHEY AND DELBRÜCK, were carried out in an attempt to elucidate the nature of cofactor activation.

MATERIALS AND METHODS

T4 bacteriophage and *E. coli*, strain B[2] have been used in the experiments reported in this work. A special broth stock of T4, referred to hereafter as T4.38, had been prepared by single plaque isolation from the strain T4.11[6].

The *synthetic medium* employed is the salt-lactate medium F having the following composition:

	g/liter
Sodium Lactate	10
Sodium Potassium Phosphate	5
Ammonium Chloride	1
Magnesium Sulfate ($7H_2O$)	0.1
Calcium Chloride ($6H_2O$)	0.02

The *complete medium* was Difco nutrient broth, which will be referred to as N medium.

The stock T4.38 gives an assay of 10^{10} particles per ml when plated on broth agar, as compared to an assay of only 10^5 particles per ml when plated on F agar. The stock contains, therefore, no more than a small fraction of sufficient individuals. Prior to activation experiments, it is necessary to dilute the stock by a factor of at least 1:200 in F medium in order to eliminate any activity due to the cofactors present in broth.

For adsorption of the phage, unless otherwise stated, a *standard bacterial suspension* was used, prepared from a 24 hr. culture of *E. coli*, grown in F medium at 37° C with aeration, washed and resuspended in a twenty-fold volume of fresh F medium. Such a suspension contains approximately $2.5 \cdot 10^8$ viable cells per ml.

Unless otherwise stated, experiments were conducted at 15° C in a constant temperature bath, and all tubes and solutions were equilibrated at this temperature for at least half an hour before use.

Two methods were employed to measure the extent of phage-host adsorption[6]:

1. The bacteria are sedimented from the adsorption mixture by centrifugation and the free phage remaining in the supernatant, as well as an aliquot of the original mixture, are assayed on N agar. This method offers the greatest precision when most of the phages have been adsorbed.

2. Aliquots of the adsorption mixture are plated on F and N agar. The ratio of the numbers of plaques formed by the two platings, called F/N count, represents the fraction of phage adsorbed[1]. This method is suitable when only a small percentage of the phages have been adsorbed to bacteria, and when the multiplicity of infection is much below unity.

The second of the methods for estimating adsorption of cofactor-requiring phages described above, *i.e.* the F/N count, is possible because phages adsorbed on bacteria form plaques on minimum medium, while free virus does not[1]. The propagation on minimum medium of the progeny of deficient phages, freshly liberated from infected bacteria, is not due to the selection of sufficient mutants, and the plaques formed are of the same size and contain the same number of viruses as plaques formed by sufficient phages[5]. A recent direct study has shown that the phage progeny of a bacterium infected by a cofactor-requiring phage emerges from the host cell in a state in which it does not require the presence of cofactors for adsorption. This "active state" is transient like that of tryptophan activated phage, but the decay of this activity is much slower than that of activity induced by L-tryptophan (WOLLMAN AND STENT, unpublished).

ADSORPTION RATES

The phage stock T4.38 requires cofactors to be adsorbed to the host cells. A quantitative estimation of the effects of cofactor on adsorbability of the phage is obtained by measurement of the *rates of adsorption* of samples of this stock in nutrient broth and in synthetic medium supplemented with various cofactor concentrations. Suspensions of bacteria and of phage in broth or in synthetic medium containing certain amounts of tryptophan are mixed, samples are taken at various time intervals and the proportion of adsorbed phage determined by one of the methods described above.

Procedure:

Immediately before the start of the actual adsorption, 0.1 ml of a tryptophan solution is added

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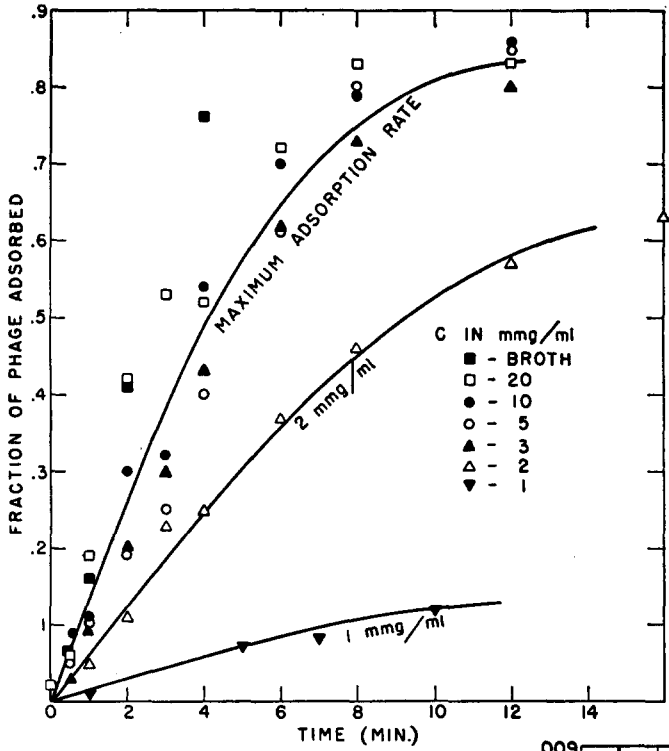

Fig. 1. Rates of adsorption of T4 phage at 15° C in broth, and in F medium supplemented with 1, 2, 3, 5, 10, and 20 mmg/ml of tryptophan. Bacterial conc.: $2.5 \cdot 10^8$ cells/ml

to 1.8 ml of a standard bacterial suspension to give a tryptophan concentration c. At $t = 0$, this mixture is poured into a test tube containing 0.1 ml of a dilute suspension of T4.38 pre-incubated with tryptophan of the same concentration c. At $t = t$, 18 ml of ice-cold F medium are poured into the adsorption mixture, and the cells of an aliquot sedimented by centrifugation. The supernatant is then assayed on N agar, and the whole adsorption mixture assayed on F and on N agar.

The results of these experiments are presented in Figs. 1 and 2, where the fraction of the phage adsorbed is plotted against the time. The nature of the cofactor requirement may be seen clearly: no perceptible adsorption takes place at tryptophan concentration below 0.25 mmg/ml, but the rate of adsorption may be increased to that found in nutrient broth by addition of sufficient cofactor.

The fraction of phage adsorbed appears to be initially proportional to time, in accordance with the kinetics of bacteriophage adsorption described previously[8, 9]. The adsorption rate constant, r, is a function of the tryptophan concentration c. Values of $r(c)$, computed by division of the initial slopes of the curves of Figs. 1 and 2 by the bacterial concentrations, are listed in Table I. A marked dependence of the adsorption rate constant on c at low c and independence of c at high c is found. The maxi-

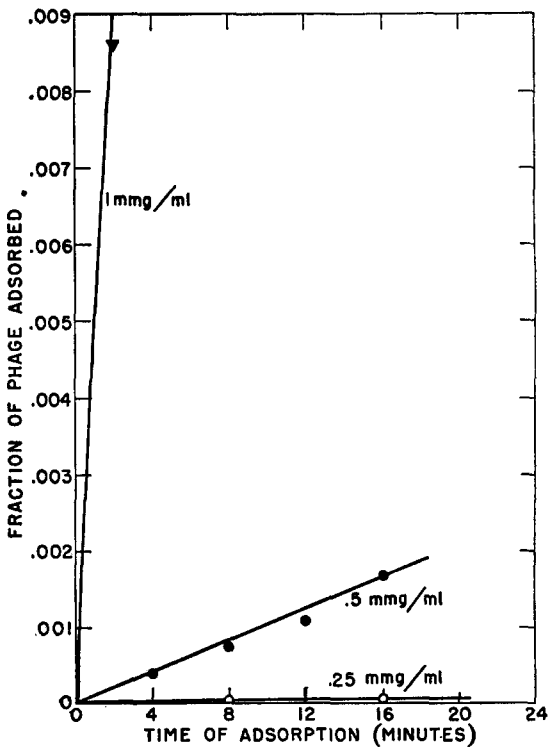

Fig. 2. Rates of adsorption of T4 phage at 15° C in Fmedium supplemented with 0.25, 0.5, and 1 mmg/ml of tryptophan. Bacterial conc.: $2.5 \cdot 10^8$ cells/ml

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mum rate of adsorption observed at high c is of the same order of magnitude as that found in nutrient broth.

TABLE I

RATE CONSTANTS OF ADSORPTION AND DEGREE OF ACTIVITY

c in mmg/ml	$r(c) \times 10^{11}$ ml/min	D (c)
0.25	<0.01	<0.0002
0.5	0.04	0.0008
1	6.0	0.03
2	25	0.5
3	50	1
5	50	1
10	50	1
20	50	1
Broth	50	—

DUMP EXPERIMENTS

In order to decide whether the cofactor interacts with the phage to make it "active" or with the bacterium to make it "sensitive", T. F. ANDERSON devised the following type of experiment, referred to as "dump experiment".

1. A large volume of phage suspended in cofactor-free medium is added to a small volume of a bacterial suspension equilibrated with cofactor, so that the cofactor concentration of the mixture is very low. After incubation of the mixture, less than 0.001% of the phage is adsorbed.

2. A large volume of bacteria suspended in cofactor-free medium is added to a small volume of a phage suspension equilibrated with cofactor, so that the resulting concentrations of cofactor and bacteria in the adsorption mixture are the same as in the above experiment. After incubation of the mixture, a considerable percentage of the phage is now adsorbed.

One may conclude, therefore, that prior exposure of *bacteria* to cofactor *does not*, whereas prior exposure of *phage* to cofactor *does* facilitate subsequent adsorption of deficient phage in cofactor-free medium. Hence the phage appears to interact with cofactor in a reversible manner, gaining or losing its "activity" upon addition or removal of tryptophan. The second type of dump experiment is interpreted to mean that a competition between adsorption and loss of activity is initiated upon dilution of the cofactor by addition of a large volume of sensitive cells, and a fraction of the phage is adsorbed before the population has lost all of its initial activity.

The activity which various cofactor concentrations impart to the phage may be studied by means of the second type of dump experiment described above by pre-equilibrating the phage with a number of different cofactor concentrations and measuring the fraction, A, ultimately adsorbed. When designing a dump experiment, it is important to make the *residual* cofactor concentration in the adsorption-deactivation mixture sufficiently low; for, if dump experiments are performed at a number of residual cofactor concentrations, c_r, of which all are known not to cause any appreciable activation of virgin phage, decreasing values of A are found until a minimum value is reached. In the experiments reported below, such minimum values occurred at $c_r \leqq 0.1$ mmg/ml.

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Procedure:

A volume of a standard bacterial suspension is poured into 0.1 ml of diluted T4.38 phage pre-equilibrated with tryptophan in concentration c. The volume of the bacterial suspension is chosen so that the resulting residual cofactor concentration is c_r. Aliquots are assayed on F and N agar plates after incubation of the mixture for ten minutes.

TABLE II

DUMP EXPERIMENTS

c in mmg/ml	Fraction of Phage Adsorbed, A		$D'(c)$
	$c_r = 0.5$ mmg/ml	$c_r \leqq 0.1$ mmg/ml	
0	0.012	0.0000097	0.00017
0.25	—	0.0000078	0.00013
0.5	0.012	0.000082	0.0014
1	0.068	0.0022	0.038
2	0.182	0.012	0.175
3	0.190	0.036	0.622
5	0.190	0.042	0.72
10	0.226	0.053	0.91
20	0.224	0.058	1

The values of A obtained in two sets of such dump experiments carried out at residual cofactor concentrations of 0.5 mmg/ml and 0.1 mmg/ml or less are presented in Table II. Although a tryptophan concentration of 0.5 mmg/ml can impart to the phage an adsorbability which is less than 0.001 of the maximum found at high cofactor concentration (cf. Table I), the values of A observed at this residual cofactor concentration c_r are very much higher than those for c_r less than 0.1 mmg/ml. The peculiar property of low cofactor concentrations to retard loss of activity, responsible for this effect, was discovered by HERSHEY AND DELBRÜCK (unpublished, 1948) and will be discussed in some detail in the second paper of this series. The minimum values of A will be shown to be very useful measures of the activity of the phage; it may be seen that they exhibit the same marked dependence on c at low c and the same relative independence of c at high c as did the rates of adsorption presented in Table I.

DEGREE OF ACTIVITY

We now formulate a measure of the extent to which cofactor has activated the phage on the basis of the adsorption rate experiments described above. The phage is thought to be "fully active" when being adsorbed to sensitive cells at the maximum rate characteristic of suspensions in high cofactor concentrations or in broth. We define the *degree of activity*, $D(c)$, as the ratio of the adsorption rate constant at c to the maximum adsorption rate constant at high cofactor concentrations, *i.e.*

$$D(c) = r(c) / r_{max} \tag{1}$$

The rate of adsorption may now be written in terms of r_{max} and $D(c)$

$$dP/dt = r_{max}\, D(c) B\, (1-P) \tag{2}$$

where P is the fraction of the phage population adsorbed and B the bacterial concentration.

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The degree of activity at various cofactor concentrations has been computed from the values of the rate constants of Table I, and is listed there in the last column. $D(c)$ is also presented graphically in Fig. 3 in a double logarithmic plot. The slope of the straight rise is 4.6.

Another definition of activity, due to T. F. ANDERSON, utilizes the technique of dump experiments as basis for quantitative formulation. The phage is defined as fully active when in a series of dump experiments at different cofactor concentrations of pre-equilibration, the adsorbed fraction, A, has reached the maximum value. How near to full activity a given cofactor concentration has brought the phage population is expressed by the ratio of A at this concentration to the maximum fraction adsorbable at excess pre-equilibration cofactor concentration. The values of this ratio, $D'(c) = A/A_{max}$ calculated from the data of Table II, are listed in the last column of Table II and presented graphically in Fig. 3 for comparison with the values of $D(c)$ computed from adsorption rate measurements.

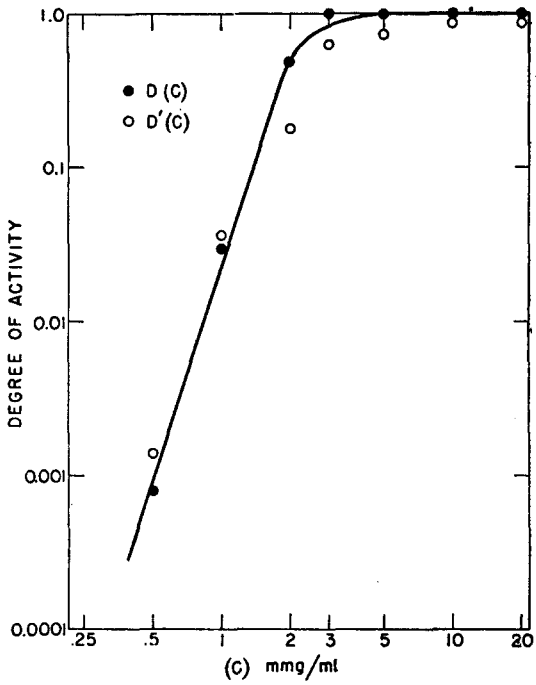

Fig. 3. The degree of activity imparted to T4 phage by a tryptophan concentration c, as measured from rates of adsorption, $D(c)$ • and as measured from dump experiments, $D'(c)$ o (15° C)

The two independent methods of estimation of activity are in reasonable agreement. Before examining whether and under what conditions $D'(c)$ and the degree of activity $D(c)$ measure the same property of the phage, it is necessary to examine the kinetics of deactivation of the cofactor-activated phage, the reaction competing with adsorption in the dump experiment.

RATE OF LOSS OF ACTIVITY

The rate at which activity is lost may be determined by a modification of the dump technique described above. The phage is first equilibrated with enough cofactor to impart to it full activity. A large volume of cofactor-free medium is then poured into the mixture to dilute out the cofactor to a low concentration. At various times after this dilution, a sample of the deactivating phage is diluted into a large volume of a standard bacterial suspension. The fraction A of the phage ultimately adsorbed is determined as in a dump experiment.

Procedure:

10 ml of F medium are poured into 0.1 ml of diluted T4.38 pre-equilibrated with 20 mmg/ml tryptophan. After t min, 0.1 ml of this further dilution is pipetted into a standard bacterial suspension. The mixture is assayed on F and N plates after 10 min incubation.

The results of this experiment are presented graphically in Fig. 4 where log $A(c,t)$

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has been plotted against the time. The fraction ultimately adsorbed decreases exponentially with the time, t, elapsed between dilution of the cofactor and addition of phage to bacteria. Hence we may write

$$A(c, t) = A(c, O)\, e^{-q_d t} \qquad (3)$$

where q_d is a rate constant of deactivation, equal to 1.2 min^{-1} in these experiments.

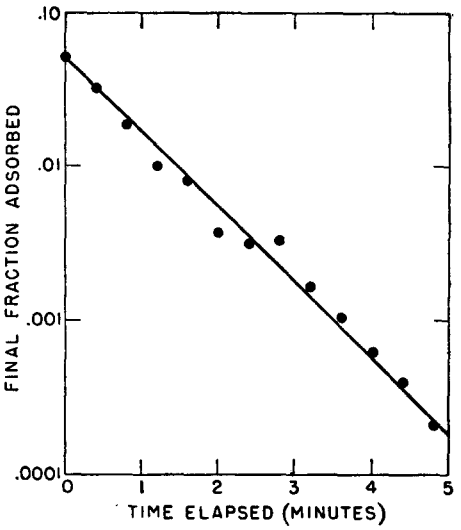

Fig. 4. The rate of loss of activity of fully activated T4 phage as measured by the decrease of the final fraction adsorbed with the time elapsed between dilution of cofactor and addition of bacteria to the phage (15° C)

EQUIVALENCE OF $D(c)$ AND $D'(c)$

In the dump experiments reported above, a maximum of about 5% of the phages was adsorbed while 95% or more had lost their activity. Therefore the rate of adsorption of phage to bacteria is low compared to the rate at which the phage population loses activity. Using equation (2) and neglecting the term $(1-P)$, we may write for $A(c)$, the final fraction of phage adsorbed,

$$A(c) = r_{max}\, B \int_0^\infty D(c, t)\, dt \qquad (4)$$

where $D(c, t)$ must now be considered as a function of the cofactor concentration during pre-equilibration as well as of the time elapsed since dilution. From the deactivation experiment we know, however, that $A(c, t)$ decreases exponentially with the time, t, elapsed between dilution of cofactor and addition of bacteria hence

$$A(c, t) = r_{max}\, B \int_t^\infty D(c, t)\, dt = A(c, O)\, e^{-q_d t} \qquad (5)$$

By differentiation of (5), and letting $t \to O$, one obtains

$$A(c) = (r_{max}\, B/q_d)\, D(c) \qquad (6)$$

where $D(c)$ is the degree of activity of the phage at the time of dilution of cofactor. Thus $A(c)$ is proportional to $D(c)$, and inasmuch as $D'(c)$ has been defined as A/A_{max}, both $D(c)$ and $D'(c)$ are proportional to A. Since at high cofactor concentration both $D(c)$ and $D'(c)$ are, by definition, equal to unity, *they must be equal at all c.* It must be borne in mind that $D'(c)$ is equal to $D(c)$ if and only if the following two conditions are fulfilled in the dump experiment:

1. The rate of adsorption is small compared to the rate of deactivation,
2. Deactivation proceeds in an exponential manner.

We will confine our discussion to the *degree of activity* as defined by the adsorption rate and regard the dump experiment as a method of measurement of this degree of activity.

HOMOGENEITY OF STOCK

It is now necessary to investigate whether our phage stock can be considered as

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homogeneous in its response to cofactor. If some phage particles are intrinsically less activable than others, it should be possible to effect a relative enrichment in less activable particles by adsorbing the stock to sensitive bacteria in the presence of a limiting cofactor concentration. If, on the other hand, the stock is homogeneous, the particles remaining free after such adsorption should be as activable as the original stock.

Procedure:

4.9 ml of a standard bacterial suspension are poured into 0.1 ml of a solution of 75 mmg/ml of tryptophan and the mixture poured into an equal volume of a suspension of T4.38 in 1.5 mmg/ml tryptophan. After various time intervals, a 0.1 ml sample of the adsorption mixture is diluted twenty-fold in F medium, incubated for ten more minutes, and assayed on F and N agar. After such time as the F/N count has been found to increase no further, the bacteria in the adsorption mixture are sedimented by centrifugation. The supernatant is diluted twenty-fold in medium containing 1.5 mmg/ml tryptophan, and a second adsorption experiment performed as above. When again the fraction adsorbed increases no further, the whole procedure of sedimentation of bacteria and resuspension of the supernatant in fresh cofactor is repeated a second time and a third adsorption measurement made. An adsorption experiment was carried out at 20 mmg/ml tryptophan with the original stock as control.

The results of an experiment devised to test the homogeneity of the stock are presented in Fig. 5, where the fraction of phage adsorbed in successive adsorption experiments is plotted against the time. The degree of activity at the cofactor concentration 1.5 mmg/ml was $D = 0.125$, as estimated from the ratio of the slopes of the initial adsorption curves to that of a control experiment at 20 mmg/ml tryptophan.

If the degree of activity $D = 0.125$ were indicative of a fraction of approximately 12% very much more readily activable at this cofactor concentration than the rest of the population, the first adsorption step, in which 20% of all phage were adsorbed at the initial rate, should have depleted the remaining 80% of most of this fraction. Hence in the second step, a very much reduced rate of adsorption should have been observed, and, after 20% of the input have again been adsorbed, practically no

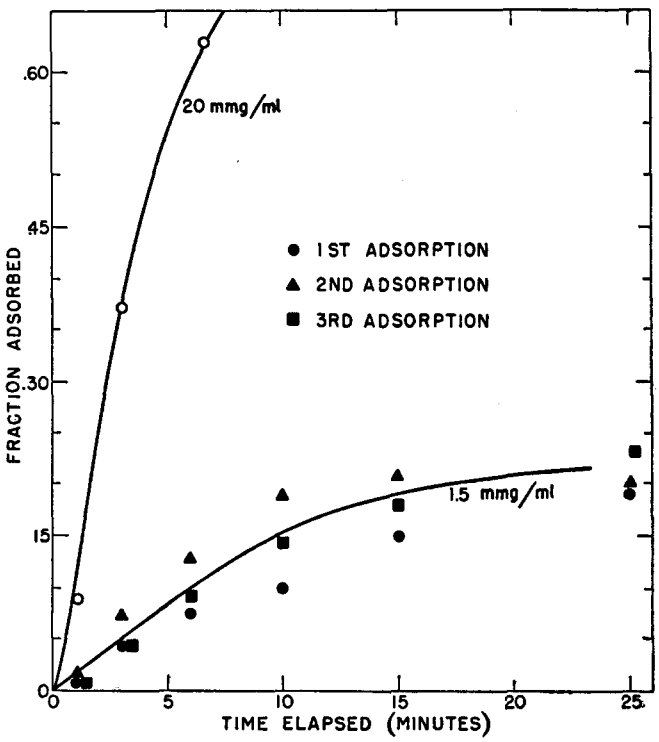

Fig. 5. The rate of adsorption of T4 phage at 15° C in F medium supplemented with 1.5 mmg/ml of tryptophan and of fractions remaining unadsorbed after two successive adsorptions, in the presence of the same concentration of tryptophan. The rate of adsorption in the presence of 20 mmg/ml as control. Bacterial conc.: $2.5 \cdot 10^8$ cells/ml

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adsorption at all should have taken place in the third step. Instead, no large differences in adsorption rates are observed in the three successive steps.

The levelling off of the adsorption curves when only 20% of the input has been adsorbed, though appearing to lend support to the idea of an heterogeneity, seems to be due to the disappearance of cofactor from the mixture by bacterial metabolism.

We may conclude, therefore, that the T4.38 stock is not a mixture of phages intrinsically differing in activability but that it is sufficiently homogeneous for $D(c)$ to express a statistical property of the phage population.

ALL-OR-NONE AND INTERMEDIATE STATE THEORIES

In order to see what statistical meaning there can be placed on the degree of activity when interpreted in terms of individual particles, it is necessary to consider two alternatives of the manner in which a phage particle can become "activated". Activation may occur in an *all-or-none* fashion, *i.e.* the particle may undergo a sudden transition from total inactivity to full activity, or in an *intermediate-state* fashion, *i.e.* the particles may pass through a spectrum of states intermediate to the extremes of the all-or-none theory. As a crude model, one may visualize the phage under the all-or-none theory as being readily adsorbable if one certain site on its surface is active. If this site is not active, the phage cannot be adsorbed at all. For the intermediate-state theory, one may think of the phage as having many such reactive sites on its surface and the ease with which the particle may be adsorbed increases with the number of sites that are active. The degree of activity assumes different meanings in these two theories.

1. *All-or-none* theory. $D(c)$ is a *distribution*, being that fraction of the phage population which is in the active state in which a particle may be adsorbed to the host cells with the velocity characteristic of that of related non-cofactor requiring strains.

2. *Intermediate-State* theory. $D(c)$ is the *property of a state* of individual phage particles. A particle in the state $D(c)$ is adsorbed to host cells under standard conditions with a probability per time unit which is the fraction $D(c)$ of the probability of related non-cofactor requiring strains.

It has thus far not been possible to ascertain which of these alternatives represents the actual mechanism of activation, but a description of the following unsuccessful attempt to make an empirical decision between them may elucidate the importance of a clear distinction between all-or-none and intermediate-state theories.

DUMP EXPERIMENT AT HIGH BACTERIAL CONCENTRATION

One of the conditions for the validity of equation (6), relating A, the fraction adsorbed in a dump experiment, to the degree of activity D is that the rate of adsorption is low compared to the rate of deactivation. One may now, on the contrary, consider the limiting value of A when the rate of adsorption becomes very large compared to the rate of deactivation. Under the all-or-none theory, the limiting value of A in such dump experiments should be D, for no matter how rapidly adsorption may proceed, the fraction adsorbed cannot exceed that fraction of the phage population which happens to be in the active state at the time of addition of the bacterial suspension. Under the intermediate-state theory, however, the limiting value of A should be unity, since the entire phage population is adsorbable at the instant of the dump and by more and more

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favoring adsorption over deactivation, a greater and greater fraction of the whole phage population should be adsorbed before activity is lost.

These concepts may be readily demonstrated analytically. Since, in contrast to the derivation pertaining to the fraction adsorbed in the previous dump experiments, deactivation is thought to be slow compared to adsorption, we may write for the rate at which phages are being adsorbed:

All-or-none	*Intermediate-state*
$dP/dt = r_{max}\, B(D-P)$	$dP/dt = r_{max}\, BD(1-P)$.

Upon integration, where D is now thought to be independent of time, and passing to the limit $t = \infty$, we find

All-or-none	*Intermediate-state*
$A = D(1-e^{-rBt}) = D$	$A = 1-e^{-rBt} = 1$,

the result deduced above.

It was hoped that by increasing the bacterial concentration in the dump mixture, it would be possible to make the rate of adsorption sufficiently large compared to the rate of deactivation to decide between the two alternative views. Dump experiments were carried out after pre-equilibration of the phage with 1 mmg/ml and, as control, with 20 mmg/ml tryptophan, using the procedure already described, but increasing the cell concentration to various multiples of that of the standard suspension.

TABLE III

DUMP EXPERIMENT WITH INCREASING BACTERIAL CONCENTRATION*

Bacterial Conc. in Multiples of the Standard Suspension ($2.5 \cdot 10^8$ cells/ml)	Fraction of Phage Adsorbed, A when pre-equilibrated with c	
	c = 1 mmg/ml $D(c)$ = 0.05	c = 20 mmg/ml $D(c)$ = 1
1	0.0075	0.15
4	0.015	0.39
10	0.019	0.42
20	0.017	0.46
40	—	0.45
80	0.013	0.41
160	0.018	—

* Bacteria in the logarithmic growth phase were employed in this experiment to avoid loss of infective centers, observed when resting bacteria were used in dense suspensions. This may account for the high values of A obtained as compared to the corresponding values in Table II[8].

The results of these experiments are presented in Table III, from which it is seen that the fraction adsorbed at the cofactor concentration of 1 mmg/ml never rises above 0.02. A, therefore, always remains below the degree of activity (D = 0.05) as required by the all-or-none theory and does not approach unity as predicted from the intermediate-state theory. No matter which of the alternative views is true, however, the fraction adsorbed at 20 mmg/ml, when D = 1, should approach unity, but, as is seen

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from Table III, it does not rise above 0.5. Hence it is apparent that some condition required for the validity of the interpretation of this experiment has not been met.

Further investigations (STENT AND WOLLMAN, unpublished) have revealed the reason for this curious behavior. It was found that the assumption of a proportionality of the adsorption rate to the bacterial concentration fails above a certain cell density. At 15° C, a stock of T4.38 phage reaches a maximum rate of adsorption of about 0.5 min^{-1} when the adsorbing bacteria are in concentration greater than 10^9 cells per ml. Substituting this maximum rate of adsorption and the rate constant of deactivation of 1.15 min^{-1} into equation (6), we find

$$A_{max} = (r_{max}\, B/k_d) = (0.5/1.15) \times 1 = 0.44$$

While this calculation satisfactorily explains the low value of A_{max}, we have no way of making an independent estimate of the maximum adsorption rate of partially activated phage. Hence, until means for either increasing the rate of adsorption or decreasing the rate of deactivation are found, it does not seem possible to use this test to discriminate between the all-or-none and intermediate state alternatives.

COFACTOR UPTAKE

An important question arising in connection with the physiological state of cofactor activated bacteriophage, as measured by the degree of activity $D(c)$, is whether and to what extent the virus particle actually combines with cofactor molecules. The conditions of the experiments presented thus far were such that the number of tryptophan molecules per unit volume of solution (10^{15} to 10^{17} molecules/ml) exceeded the number of virus particles (10^6 to 10^8 particles/ml) by many orders of magnitude. Hence, even if a large number of cofactor molecules attached themselves to individual phages, diminution of the free tryptophan in the medium would not be noticeable. To detect any possible uptake of tryptophan by virus particles, it is therefore necessary to prepare a more concentrated suspension of phage (high-titer stock).

Three liters of a growing culture of bacteria (10^9 cells/ml) in synthetic glucose medium were inoculated with an equal concentration of cofactor-activated T4.38 phage. The lysate obtained was purified and concentrated by differential centrifugation to a volume of 20 ml of final titer $2.4 \cdot 10^{13}$ plaque-forming particles per ml. The infectivity index was $10^{-15.8}$ grams of nitrogen per infective particle, showing that the major part of the suspension actually consisted of active virus particles[10]. The cofactor requirements of the new stock were essentially the same as those of the parent stock T4.38.

It was sought to estimate uptake of tryptophan by two different methods; *cofactor depletion* of a solution of tryptophan in which the high-titer stock had been suspended and *competition* of phage particles *for cofactor* in activity measurements.

1. *Depletion Experiments*

A dense suspension of bacteriophages is added to a solution of tryptophan of known concentration. The bacteriophages are then removed by filtration and the concentration of cofactor remaining in the filtrate is determined.

Procedure:

5 ml of suspensions of various concentrations (from zero to $1.2 \cdot 10^{13}$ particles/ml) of the high-

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titer stock in synthetic medium plus 4 mmg/ml tryptophan were filtered through ultra-filter membranes* at room temperature.

The first 3 ml of each filtrate were analyzed for their tryptophan content by two different methods.

a. A microbiological assay employing *Lactobacillus arabinosus*, performed on four aliquots of each filtrate corresponding to 0.75, 1.5, 3 and 6 mmg of tryptophan of the original solution.

b. A dump experiment, carried out by adding 0.1 ml of dilute T4.38 suspended in cofactor-free medium to an equal volume of each filtrate, and comparing the percent adsorbed, A, with a series of parallel dump experiments using known cofactor concentrations.

It was not possible to detect any difference between the cofactor concentrations of solutions before addition and after removal of the high-titer stock. The inaccuracies of this depletion method are such that a disappearance from the initial solution of about 1 mmg/ml of tryptophan, corresponding to a maximum uptake of 200–300 tryptophan molecules per phage particle, may have gone unnoticed.

2. *Competition Experiment*

Large volumes of bacterial suspensions are dumped into small volumes of concentrated and of dilute suspensions of the high-titer stock, equilibrated with various tryptophan concentrations, and the final fractions of phage adsorbed, A, are measured. The tryptophan concentration necessary to impart the degree of activity actually observed for the concentrated phage suspensions is then compared with the tryptophan concentration necessary to impart the same degree of activity to the dilute phage suspension. The difference between these concentrations represents the extent to which phages in the concentrated suspension have depleted the medium of cofactor.

Procedure:

2 liters of standard bacterial suspension are poured with moderate stirring into 0.1 ml of T4.38 at concentration p particles/ml pre-equilibrated with c mmg/ml tryptophan. After 10 min incubation, aliquots are assayed on F and N agar plates.

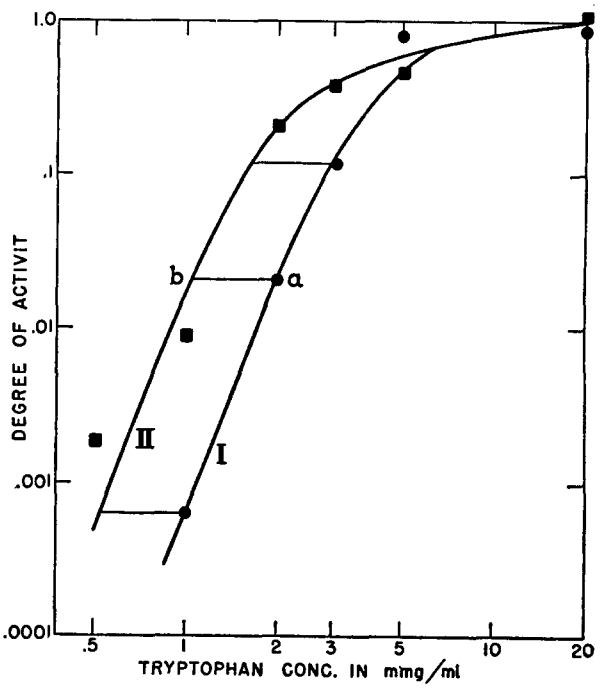

Fig. 6. Competition for cofactor. The degree of activity at various input tryptophan concentrations, as measured from dump experiments. Curve I: competitive conditions, phage concentration $1.2 \cdot 10^{13}$ particles/ml. Curve II: non-competitive conditions, phage concentration 10^8 particles/ml. The tie line a–b connects two points representing the same *available* tryptophan concentration in the medium; the difference in the abscissae of a and b indicates the tryptophan uptake by the phage

The results of this experiment are presented as curve I for competitive conditions, ($p = 1.2 \cdot 10^{13}$ particles/ml), and as curve II for non-competitive conditions, ($p = 10^8$ particles/ml) in Fig. 6 where

* These membranes were obtained through the courtesy of Dr A. GOETZ of the Physics Department of the California Institute.

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the log of the degree of activity is plotted against the log of the *input* cofactor concentration. It is seen from the divergence of curves I and II that a low cofactor concentration is able to activate a concentrated phage suspension with only reduced efficiency. At excess cofactor concentrations, on the other hand, where no competition takes place, curves I and II join. A horizontal tie-line, such as a–b, drawn between curve I and II connects two points representing the same *available* cofactor concentration in the medium. The difference in the antilogs of the abcissae of the two points divided by the concentration of phage particles per unit volume indicates the amount of tryptophan taken up per phage particle. A sample calculation based on the tie line a–b of Fig. 6 and presented in Table IV shows that at a concentration of 1 mmg/ml, corresponding to a degree of activity $D = 0.03$, approximately 200–300 molecules of tryptophan are taken up per phage particle.

TABLE IV

CALCULATIONS OF TRYPTOPHAN UPTAKE

Concentration of phage	$1.2 \cdot 10^{13}$ particles/ml
Tryptophan Conc. at point a	2 mmg/ml
at point b	1 mmg/ml
Total tryptophan uptake	1 mmg/ml or $3 \cdot 10^{15}$ molecules/ml
Tryptophan uptake per particle	$\frac{3 \cdot 10^{15}}{1.2 \cdot 10^{13}} = 250$ molecules/particle

Although this competition experiment is for technical reasons (such as presence of non-viable phage particles, aggregation, complications due to mixing, etc.) subject to a number of possible errors, it seems nevertheless to show that the bacteriophage actually combines with cofactor molecules and that the number of molecules involved, as also indicated by the depletion experiment, is not a very large one.

ACKNOWLEDGMENT

We would like to express our great indebtedness to Professor MAX DELBRÜCK for suggesting this investigation and for making available to us the results of preliminary experiments done by himself and Dr A. D. HERSHEY. His continued interest, stimulating discussions and helpful suggestions as well as the facilities of his laboratory have made possible the work reported in this and the subsequent papers of this series. We are also grateful for his critical examination of the manuscripts.

CONCLUSIONS AND SUMMARY

When adsorption rates of T_4 bacteriophages on bacteria are measured in synthetic medium to which various concentrations of tryptophan have been added, it is found that these rates are strongly dependent on the concentration of cofactor up to 2 mmg/ml and relatively independent of the concentration of cofactor above this value, a maximum rate being attained at about 20 mmg/ml. The notion of the *degree of activity* is defined as the ratio of the rate of adsorption observed at a certain tryptophan concentration c to the maximum rate. This definition is suitable for interpretations of the nature of cofactor activation but has the severe disadvantage that the phage must be at equilibrium with cofactor in the adsorption mixture.

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Since it has been shown[1] that cofactor acts on the phage rather than on the adsorption process, it is highly desirable to confine the meaning of the degree of activity to a property of the phage population itself. This is made possible by the so-called dump technique in which the outcome of a competition between deactivation and adsorption of the phage is measured by adding a large volume of bacteria to phage pre-equilibrated with cofactor.

If it is assumed that the rate constant of deactivation q_d, depends only upon the initial degree of activity and upon the medium in which deactivation takes place, it follows from equation (6) that dump experiments can be designed so as to measure the *instantaneous* adsorbability of a phage population. Such dump experiments serve as a substitute for the Gedanken-experiment of preserving the activity of the phage due to one set of conditions long enough to perform an adsorption rate measurement under another standard set of conditions. The degree of activity may now be thought of as a certain state of the phage population in which, if presented to bacteria, not necessarily in its present environment, but under *standard conditions*, it is adsorbed at a certain rate relative to the maximum rate observed under the same standard conditions. The standard conditions chosen in our experiments are a suspension of bacteria in F medium at a temperature of 15° C and a cell concentration of $2.5 \cdot 10^8$ ml^{-1}.

The dump technique makes possible the measurement of rates at which activity is gained or lost as well as investigation of interaction of phage and cofactor under various conditions of temperature, ionic strength, p_H, *etc.* which might affect the adsorption process at points unrelated to the phenomenon of cofactor activation and thus obscure the effects looked for. Such studies will be the subject of subsequent papers in this series[11, 12].

The extent to which cofactor interacts with the phage particle may be estimated by means of dump experiments employing high concentrations of phage in the activation mixture. The additional cofactor concentration necessary to bring the degree of activity of this phage up to the same level as that of a dilute virus suspension indicates the amount of cofactor taken up. Not more than 200–300 tryptophan molecules were so found to be associated with an individual phage particle when the phage had attained one-thirtieth of its maximum activity.

In attempting to recognize the meaning of the degree of activity as applied to *individual* phage particles, two principal alternatives have been considered. The *all-or-none* theory views the degree of activity as a distribution of phages among an active and an inactive class. The *intermediate state* theory envisions the degree of activity as characterizing a state of each particle intermediate to the two extreme classes of the all-or-none theory.

Although the failure of the adsorption rates of the bacteriophage to increase proportionally to the bacterial concentration prevented an experimental decision between these two alternative theories, the distinction between them retains its conceptual significance. One could, for instance, visualize the activity of an individual phage particle as being due to the fixation of a number of cofactor molecules at certain sites on the viral surface. Under this picture, the all-or-none view could mean that activation of *one* of these sites results in the activity of the whole particle. Under the intermediate-state picture, the activity of the particle would be a function of the number of activated sites on its surface.

RÉSUMÉ

1. De la relation existant entre la vitesse initiale d'adsorption du bactériophage T4, qui a besoin de cofacteur pour être adsorbé par les bactéries sensibles, et la concentration de tryptophane dans le milieu synthétique, est tirée la notion de *degré d'activité*, défini comme étant le rapport entre la vitesse d'adsorption à une concentration de cofacteur donnée et la vitesse maxima observée.

2. Après dilution brusque d'un mélange de bactériophage et de cofacteur par une suspension bactérienne, deux phénomenes se produisent concuremment: perte d'activité du bactériophage et adsorption sur les bactéries. Cette méthode d'adsorption compétitive permet de restreindre la notion de degré d'activité, qui peut être consideré comme une propriété caractéristique de la population de bactériophages, indépendante des conditions d'adsorption sur les bactéries.

3. Le lysat bactériophagique utilisé dans nos expériences est homogène en ce qui concerne son activabilité. Le degré d'activité représente donc une propriété statistique de la population de bactériophages.

4. En utilisant des concentrations élevées de bactériophages, dans des conditions telles qu'ils entrent en compétition pour le cofacteur présent, on peut estimer l'ordre de grandeur du nombre de molécules de tryptophane fixées par corpuscule bactériophage. Ce nombre est d'environ 300 (limite supérieure) pour une activité correspondant à 1/30 de l'activité maxima.

5. Deux conceptions — celle du *tout-ou-rien* et celle des *états d'activité intermédiaire*—ayant pour but de rapporter au corpus cule bactériophage la notion de degré d'activité, sont considérées.

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ZUSAMMENFASSUNG

1. Aus dem Verhältniss zwischen der Adsorptionsgeschwindigkeit des Kofaktor-erfordernen Bakteriophagen T_4 an homologe Bakterien und der Tryptophankonzentration im synthetischen Medium wird der Begriff des Aktivitätsgrades der Phagenpopulation entwickelt.

2. Wenn durch plötzliche Verdünnung mit einer Bakterienaufschwemmung aktivierten Phagen Kofaktor entzogen wird, so kann der infolge des eintretenden Wettbewerbs zwischen Adsorption und Desaktivierung an Bakterien gebundene Bruchteil der Phagen zur Messung des Aktivitatsgrades dienen. Es ist somit möglich den Aktivitätsgrad als eine Eigenschaft der Phagen-population selbst und als unabhängig von den Umständen des Bindungsvorganges zu betrachten.

3. Da das in diesen Versuchen benützte Phagenlysat sich in seiner Aktivierbarkeit als homogen erwies, stellt der Aktivitätsgrad somit eine *statische* Eigenschaft der Phagenpopulation dar.

4. Durch Verwendung konzentrierter Phagenaufschwemmungen hat sich 300 Tryptophan Moleküle pro Phage als obere Grenze der Anzahl der bei 1/30 voller Aktivität an Phagen gebundene Tryptophan Moleküle schätzen lassen.

5. Um den Begriff des Aktivitätsgrad auf einzelne Phagen Teilchen zu beziehen, wurden zwei Möglichkeiten des Aktivierungsvorganges—eine *Alles-oder-Nichts* und eine *Zwischen-Zustands* Theorie—erwogen.

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