STUDIES ON ACTIVATION OF T4 BACTERIOPHAGE BY COFACTOR

II. THE MECHANISM OF ACTIVATION

by

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INTRODUCTION

A quantitative formulation of the cofactor requirement of T4 bacteriophage was discussed in terms of the *degree of activity* $D(c)$ in the preceding paper[1]. It was shown that $D(c)$ could be recognized as a property of the phage population resulting from the interaction of phage with cofactor. We shall now attempt to arrive at a model of the activation process through a study of the kinetics of the manner in which a phage population acquires or loses its degree of activity.

MATERIALS AND METHODS

The phage stock, bacteria, media, and methods used were identical to those employed in the experiments reported in the preceding paper. The expression *standard bacterial suspension* again refers to a twenty-fold dilution of a washed and resuspended 24 hr. culture of *E. coli*, grown in F medium, containing approximately $2.5 \cdot 10^8$ viable cells per ml.

ACTIVATION KINETICS

The rate at which the degree of activity increases when a phage population is brought into an environment of greater cofactor concentration may be determined by a modification of the "dump experiment"[2]. A volume of a solution of cofactor is added to a phage suspension in cofactor-free medium to give a cofactor concentration c in the resulting mixture. After various time intervals t a large volume of a standard bacterial suspension is added, diluting the cofactor to a low residual cofactor concentration c_r. The fraction of phage adsorbed to bacteria is determined after incubation of the phage-bacteria mixture. The degree of activity D possessed by the phage at the time t is estimated by dividing the fraction of the phage adsorbed when the dumping of bacteria was made at t by the fraction adsorbed when bacteria are dumped into maximally activated phage.

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Procedure:

1.9 ml of F medium plus tryptophan in concentration c are added to 0.1 ml of dilute T4.38 stock. After t min, a volume of a standard bacterial suspension sufficiently large to reduce the residual tryptophan concentration to less than 0.1 mmg/ml is dumped into the activation mixture. Aliquots of the phage-bacteria mixture are assayed on F and N agar after incubation for ten minutes.

The results of this experiment, carried out at c = 0.5, 1, 2, 3, 5, 10 and 20 mmg/ml of tryptophan, are presented graphically in Figs. 1a and 1b, where $D(c, t)$ has been plotted against the time. Activation takes place at all cofactor concentrations without lag, the degree of activity attained after addition of cofactor rising initially proportionally to the time. The proportionality constant of this relation, or rate constant of

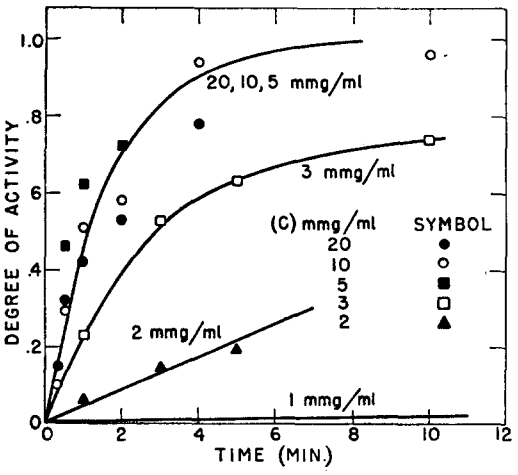

Fig. 1a. Rates of activation of T4 phage at 15° C in F medium supplemented with 20, 10, 5, 3, 2 and 1 mmg/ml of tryptophan

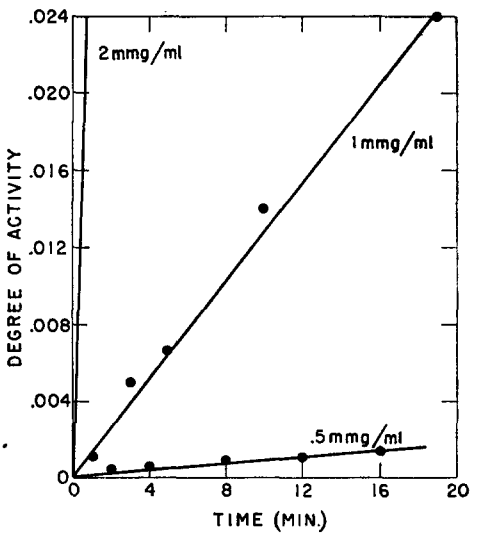

Fig. 1b. Rates of activation of T4 phage at 15° C in F medium supplemented with 1 and 0.5 mmg/ml of tryptophan

activation, q_a, has been measured from the slope of each curve, and is presented graphically in Fig. 3, where log q_a has been plotted against log c. It may be seen that q_a varies approximately with the fifth power of c at low c but levels off to a value of 0.6 per min at values of c higher than 3 mmg/ml.

DEACTIVATION KINETICS

The method for measuring the rate at which the degree of activity decreases when a fully activated phage population is brought into an environment of very low cofactor concentration has been described in the preceding paper. The cofactor with which a phage stock has been equilibrated is diluted out, sensitive bacteria are added after a time t has elapsed, and the final fraction of phage adsorbed, A, is determined. The dependence of $D(c)$ on time may then be estimated by dividing the values of A found at the various times t by the initial value of A when t = 0.

The results of a series of experiments are presented in Fig. 2 where log D is plotted

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against the time. It is seen that D decreases exponentially without exhibiting any initial lag, the rate constant of deactivation, q_d, being 1.15 per min when the residual cofactor concentration is equal to or less than 0.1 mmg/ml.

EFFECT OF RESIDUAL COFACTOR ON THE RATE OF DEACTIVATION

In dump experiments, residual cofactor concentrations c_r may affect the fraction of phage ultimately adsorbed even though the same cofactor concentrations can effect very little *activation* of virgin phage. This unexpected effect, discovered by HERSHEY AND DELBRÜCK (unpublished, 1948) was traced by them to an ability of residual cofactor concentrations to retard the loss of activity of active phage.

The rates at which the degree of activity $D(c)$, due to the presence of a fixed initial cofactor concentration c, decreases to a degree of activity $D(c_r)$ due to a series of different residual cofactor concentrations c_r has been measured. The degree of activity at any time is again estimated from the ratio of the final fractions adsorbed at $t = t$ and $t = 0$.

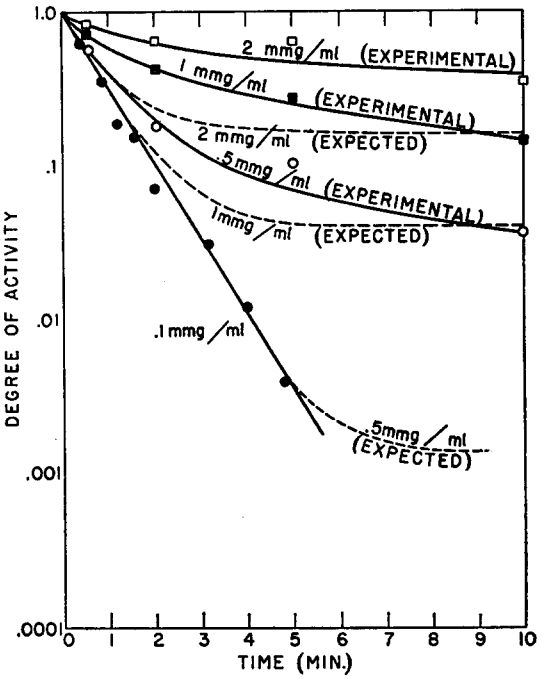

Fig. 2. The observed rates of deactivation of fully activated T4 phage at 15° C in F medium in the presence of 2, 1, 0.5, and 0.1 mmg/ml of tryptophan: in points connected by *solid* lines. The rates of deactivation as estimated on the basis of reactivation of deactivated phages, in *broken* lines.

Procedure:

A volume of F medium is poured into a test tube containing 0.1 ml of diluted T4.38 stock plus 20 mmg/ml tryptophan, so that the resulting residual cofactor concentration of the mixture is c_r. After t min, 0.1 ml of this mixture is added to a volume of a standard bacterial suspension so that the now remaining tryptophan concentration is below 0.1 mmg/ml. The adsorption mixture is assayed on F and N plates after incubation for ten minutes.

The results of these experiments carried out at $c_r = 0.5$, 1, and 2 mmg/ml tryptophan are presented graphically in Fig. 2 where log D is plotted against the time. If the effect of the residual cofactor were due solely to *re*-activation of deactivated phage, the observed net rate of deactivation would be the difference between the "pure" deactivation rate and the *re*-activation rate. Such net rates have been calculated for deactivations from full activity to the equilibrium activity at three residual cofactor concentrations $c_r = 0.5$, 1, and 2 mmg/ml tryptophan, using the value of the deactivation rate constant $q_d = 1.15$ min^{-1} found previously and the appropriate values of the reactivation rates from the activation rate constants of Fig. 3. The calculated net deactivations are presented graphically as broken lines in Fig. 2. Their initial slope is the same as that for $c_r = 0$, but they later curve to the right and approach asymptotically the respective final values of $D(c_r)$.

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The experimental points found differ in a striking manner from these calculated curves. Residual cofactor concentrations make their presence felt by slowing down the initial rate of deactivation and by altering the simple exponential character of the deactivation kinetics, since the rate decreases during the course of the process.

The log of the initial rate constants of deactivation, computed from the initial slopes of the experimental curves are presented graphically in Fig. 3 as functions of the log of the residual cofactor concentration c_r.

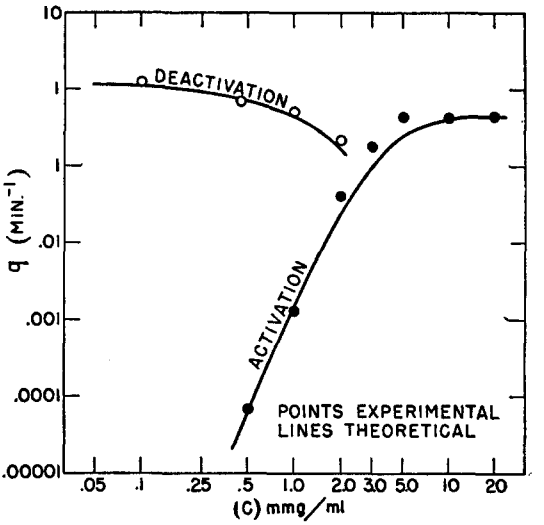

Fig. 3. The rate constants of activation and deactivation of T_4 phage at 15° C in the presence of various tryptophan concentrations c. The *points* are computed from the data of Figs. 1a, 1b, and 2. The *curves* are calculated from the equations implied by the model.

ANALYSIS OF RESULTS

1. *Absence of Lag*

Degree of activity and reaction with cofactor. The emphasis of this study has been, until this point, on the determination of a *physiological state* of the phage population, *i.e.* the degree of activity, in relation to the concentration of cofactor in the medium. To attempt an understanding of the mechanism of activation of the bacteriophage particle it is necessary to bridge the gap between the physiological and chemical concepts involved.

The degree of activity $D(c)$ increases initially proportionally to time in measurements of the rate of activation and, conversely, decreases exponentially with time in measurements of the rate of deactivation. If one visualizes the interaction of phage and cofactor during an activation experiment as resulting in a number of active sites, $S(c)$, the functional relation of $S(c)$ to time when combined with the functional relation of $D(c)$ to $S(c)$, must result in the proportionality of D to time. The simplest assumption is that of a direct proportionality between the number of active sites S and the physiological state D.

Concept of key site. The activation of individual sites must occur independently of the state of other sites since an acceleration or deceleration of the rate of change of D should be observed if the probability of activation of a site were affected by the state of other sites. We will speak of the activable sites as *key sites*, which term is to denote a region in or on the phage particle where cofactor and phage undergo reactions affecting the adsorbability of the phage particle in an all-or-none manner. Deactivation consists of the reverse of this step, *i.e.* the key site becoming inactive. *The degree of activity is then equal to that fraction of the key sites of the phage population which is in an active state.*

The key site concept can now be considered in relation to the all-or-none and intermediate-state alternatives under which a phage *particle* may be thought to become active, as discussed in the first paper of this series. Under the all-or-none theory, where individual phage particles are either active or inactive, each particle has only one such key site. Under the intermediate-state theory, where individual phages may exist in

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a variety of states of adsorbability, each particle possesses many key sites, the activity of the particle being equal to the fraction of *its* key sites in the active state.

In the following we will confine our discussion of the mechanism of cofactor activation to the reactions by which key sites become active.

2. *Rate of Activation*

Dependence on c at low c. The dependence of the rate of activation on the cofactor concentration at low c exhibited in Fig. 3 is now interpreted to mean that at low c the probability per time unit of activation of key sites is proportional to the nth power of c, n being a number near five. This suggests that the key sites are activated by complexes of n cofactor molecules. These complexes could be formed either *at the key sites* in a reaction leading rapidly to pre-equilibrium between complexes and free cofactor molecules or they could be formed *in the solution* before the phages are introduced.

Independence of c at high c. The independence of the rate of activation on the cofactor concentration at high c is interpreted to mean that the probability of activation of a key site per time unit is not increased by increasing c in this range. The key sites are then saturated with the reactive complexes of n cofactor molecules and the observed maximal rate of activation is the rate at which the complexes undergo a final reaction imparting activity to the key site. The rate of activation is a product of the rate of the final activation reaction of the complexes at the key site (which is independent of c) and the fraction of key sites occupied by complexes of n cofactor molecules (which depends on the nth power of c at low c and is independent of c at high c).

3. *Rate of Deactivation*

External cofactor can retard the rate of deactivation. This finding offers an important clue to the nature of the activation mechanism and permits a decision between the two alternative ways in which the reactive complexes of n cofactor molecules might be formed, *i.e.* as complexes in the solution or at the key site itself.

Complex formation in solution. The retardation of the rate of deactivation by the presence of residual cofactor concentrations c_r could be explained by the hypothesis of complexes of n cofactor molecules in the solution, only if the rate of formation and dissociation of such complexes is assumed to be slow. A dilution of the cofactor from c to c_r would then initially reduce the concentration of complexes by a factor of only c/c_r rather than by the nth power of this factor required by the final equilibrium concentrations. At the initial stages of the deactivation process, therefore, *re*activation would proceed at a rate much greater than that expected on the basis of the activation rates data of Fig. 3.

If such slowly dissociating complexes really existed, the rate of activation at a limiting cofactor concentration should be faster if freshly diluted cofactor rather than an aged dilution of cofactor is used. Similarly, the rate of deactivation in the presence of a residual cofactor concentration should be faster if the original cofactor is greatly diluted into medium already containing cofactor in the desired residual concentration, rather than being only moderately diluted into cofactor-free medium to provide the residual cofactor concentration c_r.

No such differences were found in experiments designed to test this point. The reactive complexes of n cofactor molecules must therefore be formed at the key sites.

Cooperation at the key site. The rate at which cofactor molecules appear or dis-

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appear at, or be "adsorbed to" and "desorbed from", the key site must be rapid with respect to the rate of activation for activation to be a pseudo-first order reaction. Since deactivation occurs at a rate comparable to that of activation, we conclude that cofactor molecules which have interacted to activate a site can no longer dissociate from it as readily as can adsorbed cofactor molecules, *i.e.* that they are "bound". We must then sharply distinguish between *adsorbed* and *bound* cofactor molecules. The rate of deactivation may be thought to be retarded by the presence of residual cofactor concentrations c_r through the ability of adsorbed cofactor molecules to take the place of a bound cofactor molecule which has just broken away from an active key site, thus preventing deactivation of the key site in question.

One may now formulate a model based on the above considerations, deduce the quantitative relations implied, and finally examine how well different experimental results can be united under the theory by assigning the best values to the empirical constants appearing in the equations.

MECHANISM OF ACTIVATION

1. *Description of Model*

Phage particles contain sites which exist in a physiologically inactive or active state. The degree of activity of a phage population is equal to the fraction of the sites in the active state. Single cofactor molecules adsorb and desorb rapidly at the site. If, due to fluctuations, there happen to be n or more cofactor molecules adsorbed at a site, they may interact in some manner to activate the site and be bound to it more firmly. If the complex of n molecules breaks up, the site loses its activity. An active site can also contain additional adsorbed cofactor molecules besides the n bound ones. If one of the bound cofactor molecules breaks loose from an active site to which an additional cofactor molecule happens to be adsorbed, the gap left can be filled instantaneously by the extra molecule, thus preventing deactivation of the site. We will call such sites "protected active sites". Those sites which have only recently lost their activity and still retain $n-1$ cofactor molecules may recapture the missing nth one from the solution and be reactivated. We will call such sites, which have no physiological activity but are more easily activable than virgin sites, "subactive sites".

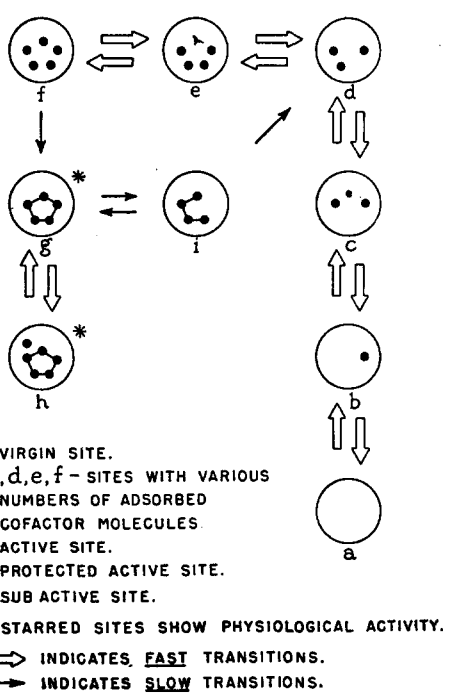

Fig. 4. Schematic representation of the model proposed for the mechanism of cofactor activation

A schematic drawing of the various alternative states of key sites and their relation to each other is presented in Fig. 4.

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2. Activation Kinetics

Let us call f_n the fraction of the key sites having n or more, and f_o the fraction having zero adsorbed (not bound) cofactor molecules. Let k_a be the rate at which the n adsorbed molecules interact to activate the site and k_d the rate at which the first of the n bound molecules of an active key space breaks loose. We assume that k_a does not depend markedly on the number of adsorbed cofactor molecules, as long as this number is at least equal to n. The fraction of the key sites becoming active per unit time is

$$k_a f_n (1-D) \tag{1}$$

The fraction of the key sites losing activity per unit time is

$$k_d f_o D \tag{2}$$

since only those sites deactivate which have no extra free cofactor molecules, *i.e.* which are not "protected". We now designate by R the fraction of subactive sites, by k_R the constant of the rate with which an external cofactor molecule may enter such key spaces to fill the breach left by the missing nth molecule, and by k_L the constant of the rate with which subactive units break up. The rate at which subactive sites are *re*-activated by the external cofactor concentration c per unit time is

$$k_{RC} R \tag{3}$$

Combining (1), (2) and (3) we find the rate of formation of active sites

$$dD/dt = k_a f_n (1 - D) + k_{RC} R - k_d f_o D \tag{4}$$

A second differential relation expresses the net rate of formation of subactive sites

$$dR/dt = k_d f_o D - (k_L + k_{RC}) R \tag{5}$$

The simultaneous equations (4) and (5) have a solution of the form

$$D = K_1 e^{-A_1 t} + K_2 e^{-A_2 t} + K_3 \tag{6}$$

The coefficients K_1, K_2, K_3 depend on the initial cofactor concentrations with which the phage had been equilibrated before the start of the experiment and on the final cofactor concentration in the presence of which the kinetics of gain or loss of activity is studied. If, for the case of a deactivation experiment, K_1 and K_2 are related to A_1 and A_2 in such a manner that D varies as $K_1 e^{-A_1 t}$ at small values of t and as $K_2 e^{-A_2 t}$ at high values of t, equation (6) describes the departure from first order kinetics observed in the presence of residual cofactor concentrations. Loss of activity commences at one rate and changes to a second, lower rate in the course of the reaction.

The form of the constants of equation (6) is, however, so complex that the precision of our experimental data hardly warrants the discussion of an exact solution of the simultaneous differential equations (4) and (5), particularly in view of the fact that two further empirical constants, k_L and k_R were introduced. Rather, after seeing that reactivation of subactive sites can account in a qualitative manner for the departure from simple kinetics caused by residual cofactor concentrations in the course of deactivation experiments, we confine our attention to *initial* rates, when $R = 0$. A much simpler differential relation remains, *i.e.*

$$dD/dt = k_a f_n (1 - D) - k_d f_o D \tag{7}$$

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Under the conditions of the activation measurements, D is near zero at the initial stages of the reaction. The term $k_a f_n$ is, therefore, the initial rate of activation q_a. Under the conditions of the deactivation experiment, D is near unity at the start, so that $k_d f_o$ is the initial rate of deactivation q_d.

3. *Dependence on Cofactor Concentration*

Since the number of cofactor molecules in the solution is large compared to the number of key sites, only a negligible fraction is to be found at key sites. We may write at equilibrium the average number of cofactor molecules per key site $\bar{c}$, as a function of the cofactor concentration

$$\bar{c} = K_c c \tag{8}$$

where Kc is the equilibrium constant of the association of single cofactor molecules with the key sites*. If a random distribution of cofactor between key sites is assumed, f_n, the fraction of the sites having n or more adsorbed cofactor molecules is proportional to the nth power of c at low cofactor concentrations and approaches unity at high c, *i.e.*

$$q_a = k_a K_c^n c^n / n! \qquad \text{(low } c\text{)} \tag{9}$$

and

$$q_a = k_a \qquad \text{(high } c\text{)} \tag{10}$$

To calculate deactivation rates in the presence of residual cofactor concentrations c_r, it is necessary to know the fraction of sites having zero adsorbed cofactor molecules, f_o, when the average number per site is $K_c c_r$. We may write

$$f_o = e^{-K_c c_r} \tag{11}$$

hence

$$q_d = k_d e^{-K_c c_r} \tag{12}$$

The observed initial deactivation rate q_d is then simply k_d for low c_r and decreases exponentially with increasing c_r.

4. *Unification of Experimental Data*

The values of $k_a = 0.44$ min^{-1} and $k_d = 1.2$ min^{-1} are obtained directly from the highest points of the respective curves of Fig. 3. By trial and error, we find as best values $n = 5$ and $K_c = 1.0$ ml/mmg. Theoretical activation and deactivation rates may now be calculated from equations (9) and (12) using these values of the empirical constants. The results of such calculations are presented graphically as solid curves on Fig. 3 for comparison with the experimental points.

5. *Equilibrium Degree of Activity*

Finally, we may obtain the dependence of the equilibrium degree of activity $D(c)$, effected by a given cofactor concentration. Setting $dD/dt = 0$ in equation (7), we find

$$D(c) = \frac{k_a f_n}{k_a f_n + k_d f_o} \tag{13}$$

* It is assumed here that saturation of key sites with adsorbed cofactor molecules occurs only at cofactor concentrations considerably greater than those considered.

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Substituting each of the two limiting cases of equations (9), (10) and (12) one finds for high c

$$D = \frac{k_a \cdot 1}{k_a \cdot 1 + 0} = 1$$

and for low c

$$D = \frac{k_a K_c^n c^n / n!}{k_a K_c^n c^n / n! + k_d}$$

Hence the degree of activity is seen to vary with the nth power of the cofactor concentration at low c, an experimental observation reported in the preceding paper. The model, furthermore shows that D approaches unity at high c in spite of the fact that the rate of activation does not increase indefinitely with c, levelling off at a maximum value which is only half that of the maximum rate of deactivation. At high c, all sites are protected and do not deactivate at all.

The values of D, calculated from equation (13) by using the rates of activation and deactivation presented in Fig. 3, are shown in Fig. 5 for comparison with the values of D found empirically in the preceding paper. It is seen that at low values of c the calculated values of D are significantly below the experimental points, though the general trend of D is unquestionably the same in both cases.

The factor responsible for the poor agreement at low c may be the neglect, implicit in equation (7), of reactivation of subactive sites. If equilibrium conditions of the more exact equations (4) and (5) are examined, one finds the relation

$$D = \frac{k_a f_n}{k_a f_n + k_d f_o (1 - k_{RC}/(k_{RC} + k_L))} \quad (14)$$

Since it was not feasible to make an evaluation of the magnitude of the constants k_R and k_L, corrected values of D cannot be computed from this relation. It may be seen, however, that the term $k_d f_o$ in the denominator is multiplied by a factor less than unity, bringing the values calculated from equation (13) towards the experimental values of D.

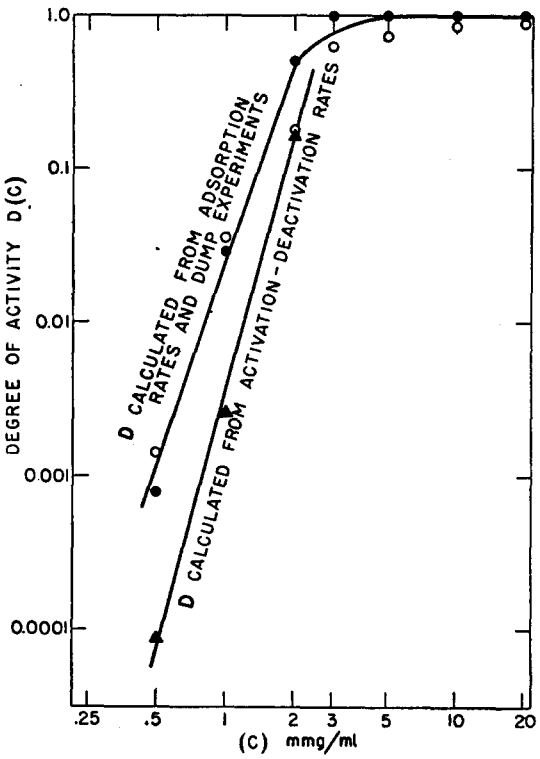

Fig. 5. The equilibrium degree of activity of T4 phage due to various tryptophan concentrations c, calculated from adsorption rates • and dump experiments o and estimated from activation-deactivation rate constants ▲

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CONCLUSIONS

The model of the mechanism of cofactor activation developed above unifies the experimental findings for which it was designed. In spite of the somewhat complicated and formal relations postulated, it would seem that no simpler picture could account for the principal features of the activation process observed. The usefulness of the model will depend on what *other* findings connected with cofactor action it can account for and what additional experiments are suggested. A study of the effect of temperature on the kinetics of cofactor activation, interpreted in terms of the model, is reported in the next paper of this series[3]. It is also hoped to re-examine the role of inhibitors such as indole[4] and that of other, less effective cofactors, such as phenylalanine, from the point of view of cooperation of four to six molecules at key sites.

SUMMARY

1. The rates of activation and deactivation of cofactor-requiring T4 bacteriophages are measured as functions of the tryptophan concentration in the medium.

2. The concept of *key-site* at which activation of the phage occurs is inferred from the kinetic data and the degree of activity of a phage population defined in terms of the state of the key sites.

3. A model of the mechanism by which key sites gain or lose activity is developed and the quantitative relations implied are derived.

4. The experimental findings of the kinetic experiments are unified by assigning suitable values to the empirical parameters appearing in the equations describing the model.

RÉSUMÉ

1. Les vitesses avec lesquelles le bactériophage T4, qui a besoin de cofacteur pour être adsorbé par les bactéries sensibles, gagne ou perd son activité, ont été mesurées en fonction de la concentration de tryptophane dans le milieu.

2. De ces résultats cinétiques est tirée la notion *d'espace critique*, siège de l'activation du bactériophage par le cofacteur. Le degré d'activité que possède une population de bactériophages est défini en rapport avec l'état dans lequel se trouvent les espaces critiques.

3. Pour rendre compte du mécanisme par lequel les espaces critiques deviennent actifs et perdent leur activité, un modèle a été établi dont les implications quantitatives sont déduites.

4. En attribuant des valeurs convenables aux paramètres qui apparaissent dans les équations décrivant le schéma proposé, il est possible de rendre compte d'une manière satisfaisante des résultats expérimentaux.

ZUSAMMENFASSUNG

1. Die Aktivierungs- und Desaktivierungsgeschwindigkeit des Kofaktor-erfordernden Bakteriophagen T4 wird bei verschiedenen Tryptophan Konzentrationen gemessen.

2. Aus den Ergebnissen kinetischer Versuche wird der Begriff von *Schlüssel-Stellen*, an welchen Aktivierung und Desaktivierung der Phagen stattfindet, abgeleitet. Der Aktivitätsgrad einer Phagenpopulation wird durch den Zustand der Schlüssel-Stellen definiert.

3. Ein Modell des Vorgangs, durch welchen Schlüssel-Stellen Aktivität erreichen oder verlieren, wird vorgeschlagen und die dadurch bedingten quantitativen Beziehungen abgeleitet.

4. Durch Verwendung geeigneter Werte für die in der analytischen Modellbeschreibung erscheinenden empirischen Grössen können die kinetischen Versuchsergebnisse gut vereint werden.

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