STUDIES ON ACTIVATION OF T4 BACTERIOPHAGE BY COFACTOR

III. CONDITIONS AFFECTING THE ACTIVATION PROCESS

by

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INTRODUCTION

The concept of the *degree of activity* $D(c)$ as an expression of the state of cofactor-requiring T4 bacteriophage was discussed in the first paper of this series[1]. The study of the kinetics by which a phage population gains or loses this degree of activity and a model of the activation mechanism based on these findings were the subject of the preceding paper[2]. The experiments on which these conclusions were based were performed under certain standard conditions of temperature and pH.

It has been shown[3], however, that modifications of the temperature and of the pH of the medium in which T4 bacteriophage has been incubated with a certain concentration of tryptophan influences the activity imparted to the phage. The activity is likewise affected by variations in ionic strength of the activation medium (DELBRÜCK AND STENT, unpublished).

The object of the present investigation has been the study of the temperature dependence of the principal features of the activation process in order to see whether such findings could be fitted into the framework of the model. It has also been attempted to interpret other pertinent facts concerning cofactor-requiring strains in terms of the proposed mechanism.

MATERIALS AND METHODS

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

RATES OF ADSORPTION

That temperature has a striking influence on the activability of T4.38 phage by low cofactor concentrations may be seen most clearly by comparing the temperature

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dependence of adsorption rates. Measurements of the rates of adsorption in the presence of excess and of limiting amounts of tryptophan were carried out at a number of different temperatures.

Procedure:

Immediately before the start of the actual adsorption, 0.1 ml of a tryptophan solution is added to 1.8 ml of a standard bacterial suspension at the temperature T to give a tryptophan concentration c. At $t = 0$, this mixture is poured into 0.1 ml of a dilute suspension of T4.38 preincubated at T with tryptophan of the same concentration c. After t min, aliquots of the adsorption mixture are diluted and assayed on F and N agar.

The results of a set of adsorption experiments carried out at $c = 1$ mmg/ml and $c = 100$ mmg/ml are presented in Fig. 1 where the log of the adsorption rate constant is plotted against the reciprocal of the absolute temperature. It is seen that the rates of adsorption at the two cofactor concentrations are nearly the same at 37° C but differ by a factor of 1000 at 3° C.

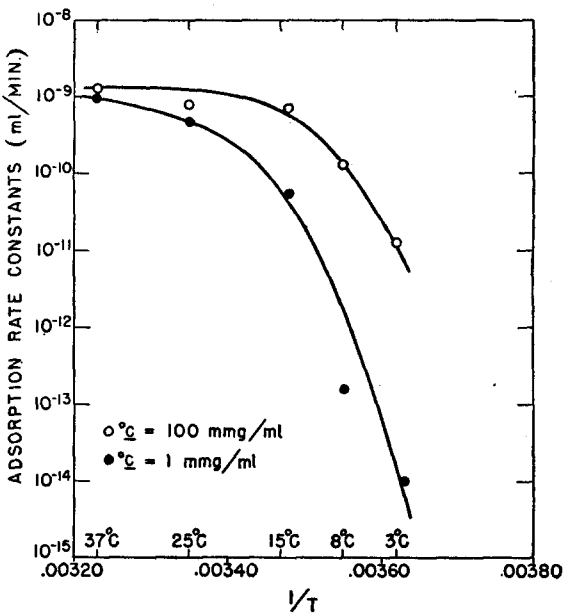

Fig. 1. Rates of adsorption of T4 phage as function of temperature, in the presence of 1 mmg/ml (•) and 100 mmg/ml (o) tryptophan. Bact. conc: $2.5 \cdot 10^8$ cells/ml

To study quantitatively this great reduction in activability conditioned by lower temperatures, it is necessary to correct the observations for the effect of temperature on the adsorption process itself. It was shown in the first paper of this series that the so-called "dump experiment", in which the degree of activity is estimated from the outcome of a competition between adsorption and deactivation, may be used for determining the adsorbability possessed by a phage population under *one* set of experimental conditions while presented to sensitive bacteria under *another* set of conditions. The temperature dependence of activation may be divorced from that of the adsorption process by varying the temperature at which the phage first encounters cofactor but keeping *constant* the temperature at which the adsorption-deactivation competition of the dump takes place. We have chosen 15° C as *standard temperature* for this purpose.

EQUILIBRIUM DEGREE OF ACTIVITY

It has been found in equilibrium measurements performed at 15° C that the degree of activity depends on the 5th power of the cofactor concentration c at low c. The efficiency with which cofactor can impart activity to the phage at various temperatures T can be estimated by equilibrating a small volume of the phage with cofactor at the temperature T and dumping into this volume a standard suspension of sensitive cells

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at the temperature of 15° C. The degree of activity $D(c.T)$ as function of c and T is then equal to the ratio of the phage adsorbed to the maximum fraction adsorbable in any dump experiment in which the adsorption-deactivation takes place at 15° C.

Procedure:

A volume of a standard bacterial suspension at the temperature of 15° C is dumped into 0.1 ml of diluted T4.38, pre-equilibrated with tryptophan in concentration c at the temperature T, so that the resulting residual cofactor concentration is less than 0.1 mmg/ml. Aliquots are assayed on F and N agar after 10 min incubation of the mixture at 15° C.

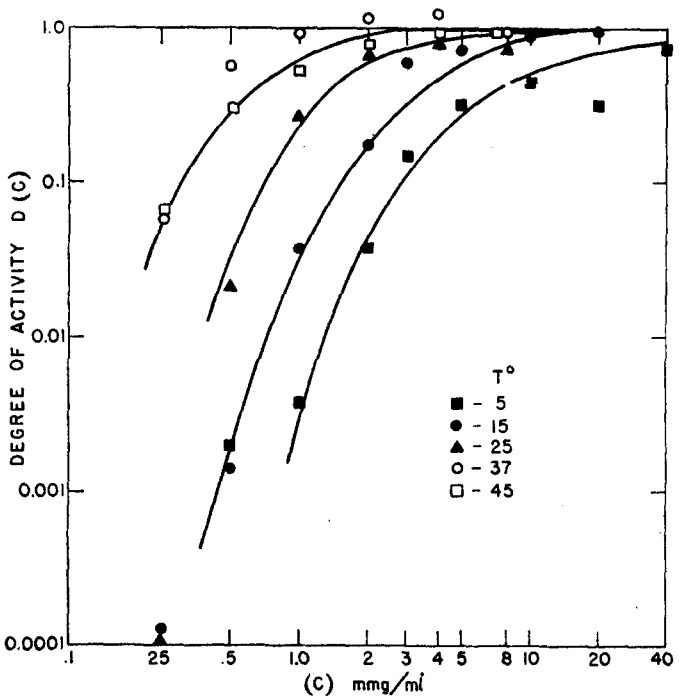

Fig. 2. The degree of activity imparted to T4 bacteriophage at different temperatures by various concentrations of tryptophan

The results of a series of dump experiments carried out at $T = 5°$ C, 15° C, 25° C, 37° C and 45° C are presented in Fig. 2 where log D has been plotted against log c. It is seen that lowering the temperature effects a drastic reduction of the activity, which may, however, always be overcome by addition of more cofactor. At limiting cofactor concentrations, D depends on the 5th power of c at all temperatures.

ACTIVATION KINETICS

The rate at which the degree of activity increases when a phage population is brought into an environment of greater cofactor concentration at various temperatures T may be determined by a modification of the "dump" technique previously described[2,3]. It has been shown that at the temperature of 15° C the rate constant q_a of activation depends on approximately the fifth power of c at low c and becomes independent of c at high c after reaching a maximum value k_a.

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Rates of activation have now been measured in experiments in which activation proceeded at the temperature T and the degree of activity was determined at the standard temperature of 15° C.

Procedure:

Equal volumes of tryptophan solution in concentration $2c$ and dilute suspension of T4.38 in cofactor-free medium are mixed at the temperature T. After incubation at T for various time intervals, small aliquots of the activation mixture are diluted into standard bacterial suspensions at the temperature of 15° C, reducing the cofactor concentration in the adsorption-deactivation mixture to less than 0.1 mmg/ml. After 10 min incubation at 15° C, the fraction adsorbed is determined by plating aliquots on F and N agar.

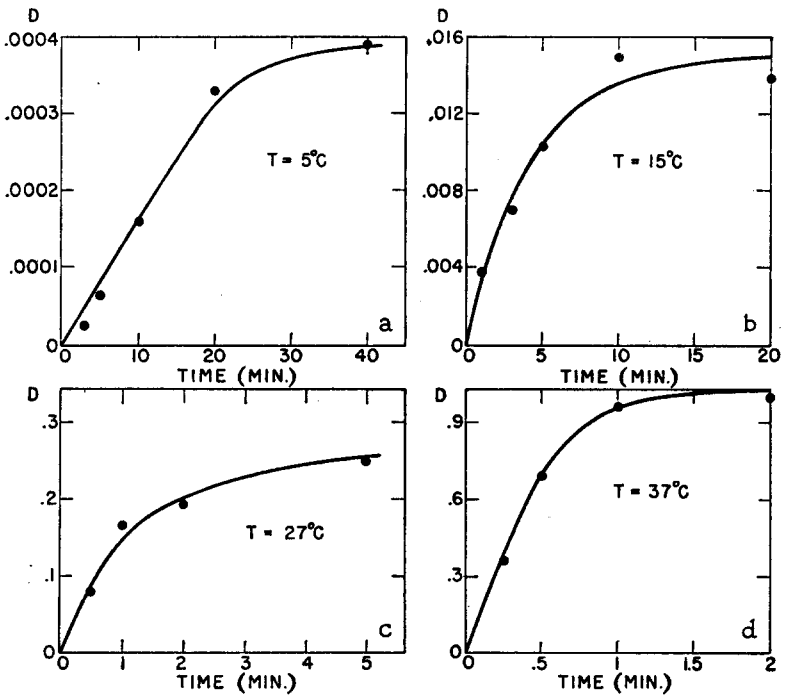

Fig. 3. Rates of activation of T4 phage at different temperatures in the presence of limiting concentration of cofactor (1 mmg/ml tryptophan). 3a: 5° C. 3b: 15° C. 3c: 27° C. 3d: 37° C.

A tryptophan concentration of 1 mmg/ml was chosen for measurement of the rate constant q_a in the range where cofactor is present in *limiting* amounts at *all* temperatures. To estimate the temperature dependence of the *maximum* rate of activation, k_a, in the presence of *excess* cofactor, rate measurements were made at a tryptophan concentration of 100 mmg/ml. The results of two such series of activation rate measurements carried out at 5° C, 15° C, 27° C and 37° C for each of the two cofactor concentrations are presented in Fig. 3a, b, c, d. and in Fig. 4, where the degree of activity, D, has been plotted against the time. The logs of the values of the rate constants of activation for each of the two cofactor concentrations, obtained from the initial slopes of the curves of these figures, are plotted against the reciprocal of the absolute temperature in Fig. 6.

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It is seen that the rate constant of activation in the range where cofactor is limiting, measured at $c = 1$ mmg/ml, exhibits an extraordinarily great temperature dependence, changing by a factor of more than 10,000 in the temperature interval 5° C to 37° C. The constant of the maximum rate of activation, measured at 100 mmg/ml, on the other hand, varies with temperature to a much lesser extent, changing by a factor of less than 50 in the same temperature interval.

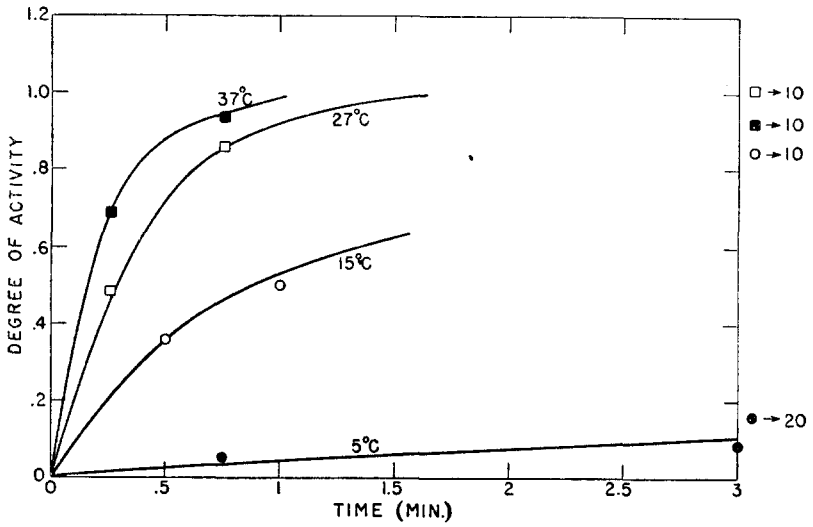

Fig. 4. Rates of activation of T4 phage at different temperatures in the presence of excess cofactor concentration (100 mmg/ml tryptophan)

DEACTIVATION KINETICS

Another modification of the dump technique can be used 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 at various temperatures T. Rates of deactivation were measured in experiments in which deactivation proceeded at the temperature T and the degree of activity was determined at the standard temperature of 15° C.

Procedure:

10 ml of F medium at the temperature T are added to 0.1 ml of a dilute suspension of T4.38, equilibrated with cofactor in concentration c at the same temperature. After incubation at T for various time intervals, small aliquots are pipetted into standard bacterial suspensions at the temperature of 15° C bringing c_f to a low level. After 10 min incubation at 15° C the fraction adsorbed is determined by plating aliquots on F and N agar.

In a series of deactivation experiments at the temperatures 5° C, 15° C, and 23° C, the phage had been brought to full activity by equilibration with 40, 20, and 10 mmg/ml of tryptophan respectively. The hundredfold dilution initiating the deactivation reaction presumably sufficed at each temperature to reduce the residual cofactor concentration c_f below that level at which it can affect the deactivation velocity in the manner discussed in the preceding paper. The results of this series are presented graphically in Fig. 5 where the log of the degree of activity D is plotted as a function of time. The

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logs of the deactivation rate constants k_d, calculated from the initial slopes of the curves of Fig. 5 are plotted against the reciprocal of the absolute temperature in Fig. 6. It is seen that the rate constants of deactivation depend on the temperature in much the same manner as do the *maximum* rate constants of activation, k_a.

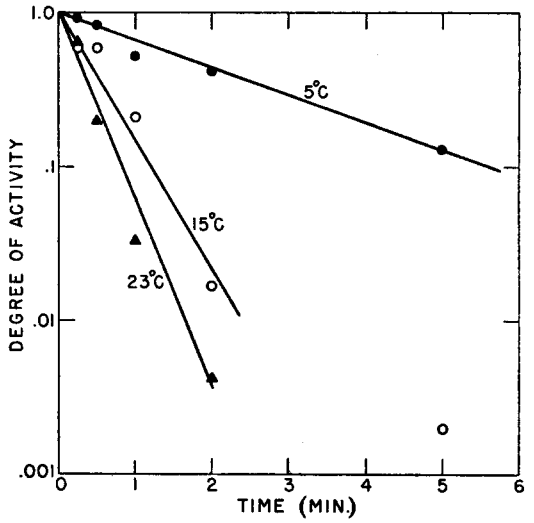

Fig. 5. Rates of deactivation of fully activated T4 phage at different temperatures. 5° C • 15° C o 23° C ▲

ANALYSIS OF RESULTS

1. *Review of Model*

Let us now examine the results of the above temperature experiments in the light of the pertinent features of the model proposed in the preceding paper. Phage particles are thought to contain *key-sites* which exist in a physiologically active or inactive state, the degree of activity of a phage population being equal to the fraction of key-sites in the active state. Single cofactor molecules adsorb or desorb rapidly at the sites, the equilibrium constant of this reaction K_c being defined such that when the cofactor concentration is c, the average number of tryptophan molecules per key-site is $K_c c$. A certain fraction of the key-sites, f_n, will, due to fluctuations, have adsorbed n or more cofactor molecules, which may then interact in some manner, with a probability k_a per unit time, to activate the site. These molecules will then be bound to the site. The bound complexes of n molecules break up again leading to loss of activity, with a probability k_d per unit time. Deactivation is affected by the presence of residual cofactor concentrations through the ability of recently deactivated sites still containing n—1 cofactor molecules to be reactivated.

At low cofactor concentrations, the rate constant of activation q_a was shown to be

$$q_a = k_a K_c^n c^n / n! \qquad (1)$$

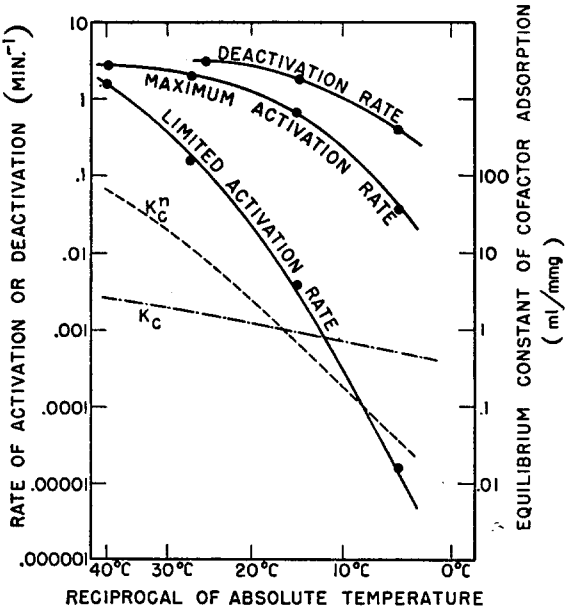

Fig. 6. Rate constants of deactivation, maximum activation (in the presence of 100 mmg/ml tryptophan), and limited activation (in the presence of 1 mmg/ml tryptophan) as function of temperature (left ordinate). Graphical computation of temperature dependence of equilibrium constant of cofactor adsorption to key-sites, K_c, (right ordinate)

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whereas at high cofactor concentrations

$$q_{a\,\max} = k_a \tag{2}$$

The rate constant of deactivation q_d may be written

$$q_d = k_d \tag{3}$$

provided that the residual cofactor concentration in the deactivation mixture is sufficiently low. Neglecting so-called "subactive sites", the equilibrium degree of activity D is

$$D(c) = q_a/(q_a + q_d) \tag{4}$$

2. *Effects of Temperature*

Since there is at every temperature a cofactor concentration range in which $D(c)$ varies with c with the same high power n, it is evident from equations (1), (3), and (4) that the basic feature of the kinetics of activation of key-sites namely, interaction of n cofactor molecules, remain unaltered by changes in temperature.

The finding that the temperature dependence of the maximum rate of activation k_a is of the same order of magnitude as that of the rate of deactivation k_d supports the idea that deactivation of a key site consists of breakage of the bond formed when the n cofactor molecules interacted.

Examination of equation (1) reveals that any change in K_c with temperature, would effect a change in q_a proportional to its nth power. The extreme temperature dependence of the rate of activation q_a when cofactor is in limiting concentrations as compared to the moderate temperature dependence of q_a in the presence of excess cofactor can now be understood. At high c, $q_{a\,\max}$ varies with temperature as k_a, the rate of formation of bound complexes at sites saturated with adsorbed cofactor molecules, whereas at limiting c, q_a, changes with the *product* of variations of k_a times the nth-powered amplification of variations in K_c, the adsorption-desorption equilibrium between cofactor molecules in solution and at key-sites.

Solving equations (1) and (2), for K_c, one finds that

$$K_c = (1/c)\left[n!\, q_a/q_{a\,\max}\right]^{1/n} \tag{5}$$

We may, therefore, obtain log K_c (1 mmg/ml, T), *i.e.* the dependence of the log of the association equilibrium constant at $c = 1$ mmg/ml on temperature, from the activation rate data presented on Fig. 6. It is only necessary to subtract the ordinates of q_a(100 mmg/ml) from those of q_a(1 mmg/ml), adding log $n!$ and dividing by n. The results of such computations are presented as broken lines on Fig. 6 where values of log K_c^n and log K_c are plotted against the reciprocal of the absolute temperature. It is seen that a moderate temperature sensitivity of K_c suffices to account for the vigorous temperature dependence of q_a in regions of low c.

3. *Heat of Adsorption*

The reduced activability of T4.38 at lower temperature has thus been traced back to a change in the affinity of tryptophan molecules to the key-sites. It appears surprising that this affinity is *lower* at lower temperatures, contrary to the usual adsorption-desorption equilibria in which the affinity of the adsorbent for the adsorbate is *greater* at lower temperatures. Using the VAN 'T HOFF equation relating the change in equilibrium constant with temperature to the heat of reaction, one may calculate from the data of Fig. 6 that approximately 4 kcal are taken up per mole of tryptophan adsorbed to key-sites. It is inferred, therefore, that the bond energies with which tryptophan molecules

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are held to water in their solvated state is by 4 kcal/mole greater than the energy of the bonds formed when such molecules are adsorbed to the key-site.

An interesting consequence of the sign of this heat of reaction is the implied *increase* in entropy attending the transfer at equilibrium of a tryptophan molecule in the solution to the key-site. From Fig. 6 one may find the equilibrium constant of this reaction $K_c = 1$ ml/mmg at 15° C or, using $3 \cdot 10^{-16}$ ml as the volume of a phage particle and 204 as the molecular weight of tryptophan, the units of K_c may be converted

$$K_c = 1 \text{ ml/mmg} = 1 \cdot \frac{3 \cdot 10^{-16}}{\frac{10^{-6} \cdot 6 \cdot 10^{-23}}{204}} = 1 \text{ phage volume per tryptophan molecule.}$$

Hence, at equilibrium, there is on the average one molecule of tryptophan per key-site when the concentration of tryptophan in the *medium* is such that there is one molecule per volume equivalent to that occupied by a phage particle. Presumably a key-site represents only a small fraction of the total volume of the phage. It is therefore astonishing that an *increase* in entropy is observed to attend the confinement of a molecule to a more restricted space. The high degree of order imparted to neighboring water molecules by solvated, ionized α-amino acids and the consequent increase in entropy if the dipole character of the ion is removed[4] might possibly account for this behaviour.

It would be of interest to compare with the findings reported here other data pertaining to the temperature dependence of the adsorption of amino acids to proteins in an appraisal of whether the situation encountered is a general one.

GENERAL CONSIDERATIONS

1. *Other Factors Affecting Activation Process*

Previous investigations have shown that factors other than temperature may influence the activation process. It was found by ANDERSON[3] that the activity imparted to T4 phage by 20 mmg/ml tryptophan increases by a factor of more than ten when the pH of the activation mixture was varied from 4 to 8. Reductions in ionic strength of the buffer of the activation mixture were observed to facilitate greatly the ability of low cofactor concentrations to impart activity to T4.38 phage (DELBRÜCK AND STENT, unpublished).

Amino acids other than tryptophan, principally phenylalanine and tyrosine as well as some analogues of tryptophan, are capable of causing activation of cofactor requiring T4 strains, although a concentration of these substances higher than that of tryptophan is necessary for achieving an equivalent activity[5, 6]. The D-isomer of tryptophan, on the other hand, appears to be completely incapable of imparting activity. DELBRÜCK[6] discovered that the presence of indole inhibits the ability of tryptophan and of phenylalanine to activate some cofactor requiring T4 strains while not affecting the adsorbability of related, non-cofactor requiring strains.

In the light of the key-site concept, an increased or decreased efficiency of activation may be due to a number of different causes. The rate constants k_a and k_d with which n cofactor molecules interact or break up at the key-site may have changed, or the number n of cofactor molecules necessary for activation of the key-site, or the affinity of cofactor molecules for the key-site, expressed by K_c, or, finally, the adsorbability of phage to bacterium, promoted by a key-site which has been activated, may be altered.

It should, in principle, always be possible to distinguish experimentally which of these aspects of activation has been affected, but the data thus far obtained do not permit such an analysis.

2. *Mutant Strains of T4*

Strains of bacteriophage T4 with different cofactor requirements for adsorption

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arise by mutation, and such strains have been described. Some mutants of T4, for instance, may require a higher or a lower concentration of tryptophan than T4.38 to achieve a given degree of activity. Different mutants might be activated most efficiently by amino acids other than tryptophan or require the presence of additional cofactors besides tryptophan, such as the calcium-requiring strains reported by DELBRÜCK[6].

These differences may now be interpreted as *mutations having altered the key-sites of the mutant* in one or more of the properties characterized by K_c, k_a, k_d, n, or the adsorbability of the key-site in the active state.

Mutants of T4 have been described[7, 8] which, *at 37° C*, are adsorbed to bacteria in the absence of cofactor with the same rate as in broth but which, *at 15° C*, are not adsorbed at the maximum rate unless cofactor is provided. It has been suggested that such "temperature mutants" may have very low cofactor requirements (*e.g.* have a high K_c), which are met by the formation of minute amounts of cofactor produced by bacterial metabolism at 37° C. At lower temperatures the cells do not produce cofactor in sufficient quantities for activation. This possibility could be checked by adsorption measurements employing killed bacteria or bacterial extracts.

If through further experiments it should be found that temperature mutants are adsorbed at 37° C in the absence of any cofactor, then these mutants must possess key-sites for which adsorption to bacteria at high temperatures occurs irrespective of whether or not they have combined with cofactor. At low temperature adsorption takes place only through key-sites which have reacted with cofactor.

The effect of temperature on the adsorbability of key-sites might be visualized, for purposes of illustration, by imagining that reaction with cofactor reduces an energy of activation involved in the adsorption of a phage on its host cell. If the reduction in the necessary activation energy brought about by cofactor is large, then "inactive" and "active" states of the key-site will be recognizable at all experimental temperatures, as in the case of the T4.38 strain. If the differences between activity and inactivity are small, then the two states may be empirically differentiable only in adsorption experiments performed at low temperatures, as in the case of the temperature mutants.

Of those mutants of T4, as well as of the related strain T2, which do not appear to require cofactor for adsorption at any temperature, very little can be said in connection with the key-site concept. Cofactor might be bound permanently to the key-site, ($k_d = 0$), or the difference in adsorbability of key-sites with or without cofactor might be so small that even the lowest experimental temperatures would not reveal it, or, finally, such strains might have no key-sites at all, *i.e.* they do not react with the substances acting as cofactors on related deficient strains.

3. *Activity and Adsorbability*

We now consider briefly *how* the adsorbability of a phage particle may have been enhanced by reaction of the key-site with cofactor.

From our present knowledge of phage adsorption[9, 10] three features of the phage surface might be isolated conceptually as being involved in the attachment process: the chemical groups which react with the bacterium to form a bond, the surface structures permitting the specific fit necessary for the binding of the reactive groups and the electrostatic surface charge. Hence, when a phage particle *cannot* be adsorbed to the host cell, one might think of it as being deficient in one or more of these essential aspects. The role of cofactor in imparting activity to a key-site then could be conceived either as that of providing the necessary chemical groups, or as an adjustment of the specific

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structure for a better fit, or as a suitable alteration of the electrostatic charge to make possible the fitting of the phage to the complementary surfaces of the bacterium.

SUMMARY

1. The effect of temperature on the rate of adsorption, on the degree of activity and on the rates of activation and of deactivation of cofactor-requiring T4 bacteriophage is studied at different tryptophan concentrations.

2. The findings can be accounted for in terms of the model of the mechanism of activation of bacteriophage by cofactor proposed previously.

3. The effects of p_H and of ionic strength, the role of other cofactors and of inhibitors, and the characteristics of mutants of T4 of different cofactor requirement are discussed in terms of the model.

RÉSUMÉ

1. L'effet de la temperature sur la vitesse d'adsorption, le degré d'activité et les vitesses avec lesquelles le bactériophage T4, qui a besoin de cofacteur pour être adsorbé par les bactéries, devient actif et perd son activité, a été étudié en présence de différentes concentrations de tryptophane.

2. Les résultats obtenus sont en accord avec les implications découlant du "modèle" proposé pour décrire le mécanisme par lequel le cofacteur active le bactériophage.

3. L'effet du p_H et de la force ionique, le rôle d'autres cofacteurs et d'inhibiteurs du processus d'adsorption, ainsi que les caractères de mutants du bactériophage T4 qui diffèrent par leurs besoins en cofacteur, sont examinés à la lumière des implications découlant du modèle.

ZUSAMMENFASSUNG

1. Adsorptionsgeschwindigkeit, Aktivitätsgrad, und Aktivierungs- und Desaktivierungsgeschwindigkeit des Kofaktor-erfordernden Bakteriophagen T4 werden bei verschiedenen Temperaturen und Tryptophan Konzentrationen gemessen.

2. Ein bereits vorgeschlagenes Modell des Aktivierungsvorganges kann in befriedigender Weise die Ergebnisse der Temperaturversuche erklären.

3. Der Einfluss von p_H und Ionenstärke, die Wirkungsweise von anderen Kofaktoren und von Hemmstoffen, sowie die Eigenschaften von Mutanten des T4 Phagen mit verschiedenen Kofaktor Erfordernissen werden im Sinne des Modells erörtert.

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