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The *lac* Repressor–Operator Interaction

III.† Kinetic Studies

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A sensitive membrane filter assay for the *lac* repressor–operator complex has been used to study the kinetics of repressor and operator interaction. The rate of dissociation of the complex is very slow and follows first-order kinetics with a half-life of 19 minutes in our standard buffer (0·05 M ionic strength). The rate of dissociation (k_b) is rather insensitive to temperature or pH, but becomes more rapid at high ionic strength. In 0·2 M ionic strength buffer the dissociation half-life is five to six minutes. The rate of association of repressor and operator is very fast and follows second-order kinetics with the rate constant for association (k_a) being 7×10^9 M^{-1} sec^{-1} in our standard buffer. The rate of association is only slightly affected by temperature or pH, but becomes slower with increasing ionic strength. It is concluded that the association of repressor and operator is aided by electrostatic attraction between the phosphate groups of DNA and a positively charged binding site on the *lac* repressor. Over a wide range of ionic strength, the ratio k_b/k_a has been found to agree well with the equilibrium constant. The binding of repressor to operator results in an unfavorable enthalpy change; the driving force for the binding reaction comes from a large entropy increase. Binding mechanisms are discussed.

1. Introduction

The *lac* repressor has the remarkable ability to bind tightly to native DNA specifically at the *lac* operator. This has been shown conclusively and independently in two separate laboratories (Gilbert & Müller-Hill, 1967; Riggs, Newby, Bourgeois & Cohn, 1968; Riggs, Suzuki & Bourgeois, 1970*b*). An important question yet to be determined is how the *lac* repressor is able to accomplish this function. In order to investigate this novel type of protein–DNA interaction, assay methods had to be devised. Two such methods have been developed to measure repressor–DNA binding: one relies on cocentrifugation of labeled repressor with unlabeled DNA in a glycerol gradient (Gilbert & Müller-Hill, 1967); the other relies on a membrane filtration technique for detecting complexes between unlabeled repressor and labeled DNA (Riggs *et al.*, 1968). This latter membrane filter assay is relatively convenient, quick and simple. Moreover, it is extremely sensitive, permitting the easy detection of 10^{-4} μg of repressor as repressor–operator complex ($\sim 10^{-15}$ mole). This assay provides a way to determine the absolute concentration of RO§ complex present in solution, and is thus well suited for studying the chemistry of the interaction between repressor and operator,

† Paper II in this series is Riggs, Newby & Bourgeois, 1970*a*.
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§ Abbreviation used: RO, repressor–operator.

an interaction fundamentally important in the regulation of gene activity. From our equilibrium studies (Riggs *et al.*, 1970*b*) a considerable amount is now known about this interaction; e.g. the equilibrium constant is close to 1×10^{-13} M in our standard buffer, but is very sensitive to ionic strength. The effect of galactosides on this interaction has also been investigated and the *in vitro* results were found to correlate well with those obtained *in vivo* (Riggs, Newby & Bourgeois, 1970*a*). In this paper we direct our attention to the kinetic aspects of the repressor–operator interaction. How quickly does the repressor find the *lac* operator? Once bound to the operator, how stable is the complex? Our membrane filter assay has permitted us to answer these questions.

Armed with a knowledge of the kinetic aspects of the interaction, we will discuss certain models for repressor–operator binding.

2. Materials and Methods

(a) *Materials*

Highly purified (purity $\geq 50\%$) wild-type *lac* repressor was used for all experiments reported here. The purification procedure has been described previously (Riggs & Bourgeois, 1968; Riggs *et al.*, 1970*b*). The ^{32}P-labeled DNA used was extracted by phenol from $\lambda\phi 80d$ *lac* phage, a defective phage, carrying the entire *Escherichia coli lac* region. Procedures for phage growth and purification have been published (Riggs *et al.*, 1970*b*). DNA concentrations were measured spectrophotometrically at 260 nm. using an extinction coefficient of 0·02 cm^2/μg.

(b) *Buffers*

BB buffer contains: 0·01 M-magnesium acetate, 0·01 M-KCl, 10^{-4} M-EDTA, 10^{-4} M-dithiothreitol, 5% dimethyl sulfoxide, 0·01 M-Tris–HCl, pH 7·4 at 24°C, and 50 μg bovine serum albumin/ml.

FB buffer is BB buffer without either bovine serum albumin or dithiothreitol.

TKID buffer is BB buffer with 0·006 M-mercaptoethanol replacing the dithiothreitol. This change has no detectable effect on RO binding.

(c) *Methods*

Details of the membrane filter assay have been published (Riggs *et al.*, 1970*b*). In essence it consists of filtering a solution containing complexes between unlabeled repressor and ^{32}P-labeled DNA through a nitrocellulose membrane filter (Schleicher & Shuell, B-6). Free DNA passes through the filter, but DNA with RO complexes is retained. Any modifications to the basic procedure given in Riggs *et al.* (1970*b*) are given in the text or Figure legends.

(d) *Typical experiments*

(i) *Kinetics of dissociation*

Sufficient repressor is added to 1·5 μg of ^{32}P-labeled $\lambda\phi 80dlac$ DNA in 3·0 ml. of BB buffer to give approximately one-half saturation of operator with repressor. At least 5 min is allowed for equilibrium to be reached and then unlabeled DNA, sheared by passage 5 times through a 27-gauge needle, is added and sampling begun. Triplicate 0·1-ml. samples are filtered through 13-mm Schleicher & Shuell filters. The volumes can easily be increased so that 1-ml. samples are filtered through 25-mm filters.

(ii) *Kinetics of association*

Using an Eppendorf pipette, repressor is added to 0·1 μg of ^{32}P-labeled $\lambda\phi 80dlac$ DNA in sufficient BB buffer to give a final volume of 3·1 ml. Timing is begun and the solution mixed rapidly with the pipette tip. After the desired incubation period, the reaction is stopped by adding a large excess (usually 100-fold or more) of unlabeled $\lambda\phi 80dlac$ DNA, again mixing rapidly with the pipette tip. Beginning precisely 1 min after adding unlabeled DNA, triplicate 1-ml. samples are filtered through 25-mm filters.

3. Results

(a) *Kinetics of dissociation*

With our membrane filter assay for RO complex, the measurement of the rate of dissociation of RO is relatively simple. In an earlier paper (Riggs *et al.*, 1970*a*), we described one form of this experiment designed to determine the effect of galactosides on the rate of dissociation. A slightly different form of the experiment will be described here with the aim of determining accurately the rate constant characterizing the dissociation and investigating other physical-chemical aspects of the reaction.

Our basic experiment is illustrated in Figure 1. We first mixed a limiting amount of repressor with ^{32}P-labeled *dlac* DNA and allowed the equilibrium concentration of ^{32}P-labeled RO complex to form. Then a 20-fold excess of unlabeled DNA (30 to 50%

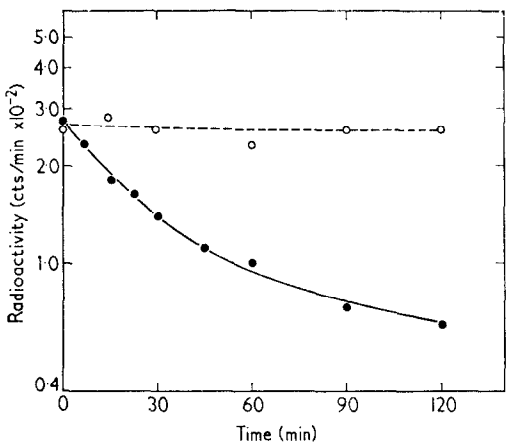

FIG. 1. Kinetics of repressor–operator complex dissociation.

Sufficient repressor was added to ^{32}P-labeled λφ80 DNA (0·5 μg/ml.; $1{\cdot}7 \times 10^{-11}$ M) to give approximately one-half saturation of operator with repressor. The buffer was TKID with 50 μg bovine serum albumin/ml. The reaction volume was 3·0 ml. and the temperature was 24°C. After 5 min during which the equilibrium concentration of ^{32}P-labeled RO complex was formed, unlabeled DNA was added to 10 μg/ml. (a 20-fold excess) and at the times indicated triplicate 0·1-ml. samples were taken, filtered through 13-mm Schleicher & Shuell B-6 membrane filters, washed once with TKID, and counted in a low-background counter. A background of 60 cts/min, due to 5% of the ^{32}P-labeled DNA being retained even in the absence of repressor, has been subtracted throughout. Each point represents the average of three filters.

– – ○ – – ○ – –, Unlabeled DNA was wild-type λφ80; —●—●—, unlabeled DNA was a mixture of λφ80 and λφ80*dlac*. The proportion of *dlac* DNA is not accurately known, but has been estimated to be between 30 and 50%.

dlac) was added and sampling begun. At each time-point the concentration of ^{32}P-labeled RO complex present in solution was assayed by filtration through a Schleicher & Shuell nitrocellulose membrane. The unlabeled *dlac* DNA will compete with the labeled DNA for the *lac* repressor and cause a reduction in the concentration of ^{32}P-labeled RO complex and a corresponding reduction in the counts retained on the filter (see Riggs *et al.*, 1970*b*). However, in order for the reduction in ^{32}P-labeled RO to occur the existing complexes must first dissociate. Thus the time-dependent decrease in counts seen in Figure 1 can be used to calculate the rate of dissociation of RO. An essential control is also shown in Figure 1; when the excess unlabeled DNA does not

contain the *lac* operator, there is no time-dependent decrease in ^{32}P-labeled RO. It is also an important fact that we have shown that an inducer, isopropyl-β-D-thiogalactoside, increases the rate of dissociation, whereas an anti-inducer *o*-nitrophenyl-β-D-fucoside decreases the rate of dissociation (Riggs *et al.*, 1970*a*).

Equilibrium studies (Riggs *et al.*, 1970*b*) have suggested that the binding of repressor to operator is a simple bimolecular reaction; i.e.

$$RO \underset{k_a}{\overset{k_b}{\rightleftharpoons}} R + O. \tag{1}$$

The differential equation describing the rate of change of ^{32}P-labeled RO should be

$$-\mathrm{d}(RO^*)/\mathrm{d}t = k_b\,(RO^*) - k_a\,(O_f^*)\,(R_f), \tag{2}$$

where k_b and k_a are the rate constants for dissociation and association, respectively; R_f is the molar concentration of free repressor; O_f^* is the molar concentration of free radioactive operator; and RO^* is the molar concentration of radioactive repressor–operator complex. Before the addition of unlabeled operator, $\mathrm{d}(RO^*)/\mathrm{d}t = 0$ and the two terms on the right side of equation (2) are equal. However, when the excess of unlabeled operator is added, the concentration of free repressor drops so that the second term becomes negligible compared to the first and

$$-\mathrm{d}(RO^*)/\mathrm{d}t = k_b\,(RO^*), \tag{3}$$

$$\ln \frac{(RO^*)}{(RO^*)_0} = \ln \frac{\text{cts/min}}{\text{cts/min}_0} = -k_b\,t. \tag{4}$$

Therefore, under these experimental conditions the decrease in RO^* should initially follow first-order kinetics. Later, as the concentration of free O^* builds up, the second term in equation (2) is no longer negligible compared to the first and the rate of decrease of RO^* would be less than expected from first-order kinetics. Eventually a new equilibrium will be reached. The results shown in Figures 1 and 2 are in agreement with the above analysis.

In Figure 2, we have concentrated on the initial linear portion of the curve, and it can be seen that first-order kinetics are followed for at least 30 minutes, with the half-life being close to 19 minutes. In addition to showing linearity, Figure 2 also illustrates that the kinetics of RO^* decrease are independent of both the initial concentration of RO^* and of the amount of unlabeled *dlac* DNA added to perturb the equilibrium. When other experiments not shown in Figure 2 are considered, the initial RO concentration has been varied from 1×10^{-11} M to $0{\cdot}5\times10^{-12}$ M and the weight excess of unlabeled *dlac* DNA has been varied from 7- to 100-fold without changing the half-life. In our standard BB buffer at 24°C, the half-life is 19 ± 4 minutes, where the range is the standard deviation for 18 experiments. From equation (4) this corresponds to

$$k_b = 6{\cdot}2 \pm 1{\cdot}3\times10^{-4}\ \mathrm{sec}^{-1}.$$

(b) *Effect of reaction conditions on* k_b

In Figure 3 are summarized the results of several experiments performed by diluting ^{32}P-labeled RO complex into buffer containing unlabeled *dlac* DNA and at the desired pH or temperature. It can be seen that the rate of dissociation is not greatly affected by pH in the range 7 to 9 or by temperature in the range 1 to 37°C. The rate of dissociation is, at the most, only 20% faster at 37°C than it is at 1°C. In contrast to the insensitivity of the rate of dissociation to the above factors, ionic strength markedly

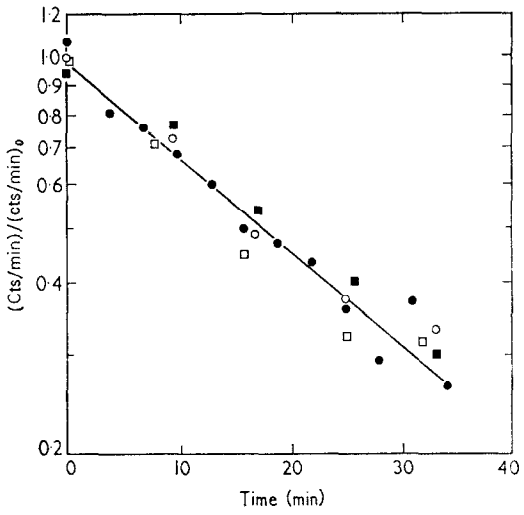

FIG. 2. Rate of dissociation of repressor–operator complex at different labeled- and unlabeled DNA concentrations.

In each case sufficient repressor was added to ^{32}P-labeled λφ80*dlac* DNA to give approximately one-half saturation of operator with repressor. Unlabeled DNA, sheared by passage five times through a 27-gauge needle, was added and sampling begun. In all cases the temperature was 23 to 24°C. The cts/min retained in the presence of 10^{-3} M-isopropyl-β-D-thiogalactoside have been subtracted throughout. In order to facilitate comparison between experiments done with widely different absolute cts/min, the results are expressed as (cts/min)/(cts/min)$_0$ where the divisor is the cts/min obtained immediately after adding unlabeled DNA.

—○—○—, BB buffer was used and the reaction volume was 2·0 ml. ^{32}P-labeled *dlac* DNA was at 0·5μ g/ml. ($1·7 \times 10^{-11}$ M). Unlabeled λφ80*dlac* DNA was added to 5·5 μg/ml. 0·1-ml. samples were filtered in triplicate through 13-mm Schleicher & Shuell B-6 filters. —■—■—, The experiment was as above, except unlabeled λφ80*dlac* DNA was added to 13·5 μg/ml. —□—□—, As above, except unlabeled λφ80*dlac* DNA was added to 40 μg/ml. —●—●—, ^{32}P-labeled *dlac* DNA was at 0·17 μg/ml. ($5·7 \times 10^{-12}$ M) and 3·1 μg of unlabeled λφ80*dlac* DNA was added. BB buffer was used and the reaction volume was 50 ml. 1-ml. samples were taken in triplicate, filtered through 25-mm Schleicher & Shuell B-6 membrane filters, and washed with 0·5 ml. of BB without bovine serum albumin.

affects k_b. This result is shown in Figure 4, where the negative logarithm of k_b is plotted against the square root of the ionic strength. RO complex dissociates faster at high ionic strength; increasing the ionic strength from 0·017 to 0·2 M results in an increase in k_b from 4×10^{-4} sec^{-1} ($t_{\frac{1}{2}} = 29$ min) to 23×10^{-4} sec^{-1} ($t_{\frac{1}{2}} = 5$ min). This result suggests that electrostatic forces contribute significantly to the binding energy.

(c) *Kinetics of repressor and operator association*

The extreme sensitivity of our assay for RO permits the rate of association of repressor and operator to be determined in a straightforward manner merely by mixing sufficiently dilute solutions of repressor and operator and following the increase in RO complex with time. Our initial experiments were done by adding repressor to a 1×10^{-12} M-solution of ^{32}P-labeled λφ80*dlac* DNA, mixing, and then at various times taking samples for filtration. When the final repressor concentration was close to 2×10^{-12} M, a time-dependent increase in RO complex was observed over a two-minute period. For the experiment shown in Figure 5, and for the other experiments reported here, one additional refinement has been made which greatly increases the accuracy

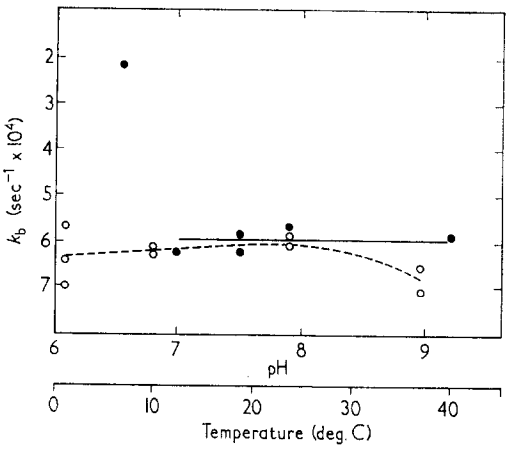

FIG. 3. Effect of pH and temperature on the rate of dissociation (k_b).

The following components were mixed in 5 ml. of BB buffer: (a) 1·3 μg of ^{32}P-labeled $\lambda\phi$80*dlac* DNA; (b) repressor calculated to be just sufficient to saturate all the operators; (c) 10 μg of unlabeled chicken blood DNA (Calbiochem), included to eliminate any binding of ^{32}P-labeled DNA not due to the *lac* repressor. After 5 min, the 5 ml. of solution now containing ^{32}P-labeled RO complexes were diluted with stirring into 45 ml. of BB buffer at the desired pH or temperature and containing 100 μg of unlabeled $\lambda\phi$80*dlac* DNA. Beginning 10 sec after diluting, triplicate 1-ml. samples were taken at 3-min intervals, filtered at room temperature through 25-mm Schleicher & Shuell membrane filters, and washed with 0·5 ml. of BB buffer without bovine serum albumin. The decrease in cts/min was followed for 30 min and k_b was calculated from the best straight line drawn through the semilog plot of the release data, as in Fig. 2. The relationship $k_b = 1{\cdot}17 \times 10^{-2}/t_{\frac{1}{2}}$ was used, where $t_{\frac{1}{2}}$ is the half-life in minutes.

--○--○--, Temperature varied; —●—●—, pH varied.

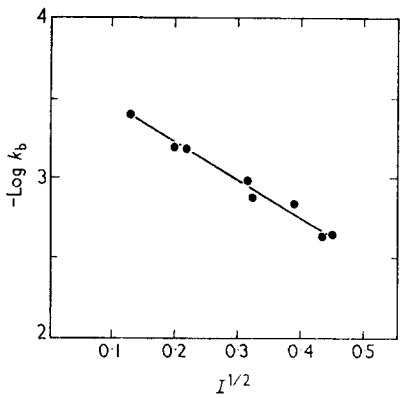

FIG. 4. Effect of ionic strength on the rate of dissociation (k_b).

Sufficient repressor was added to 0·66 μg/ml. ^{32}P-labeled $\lambda\phi$80*dlac* DNA ($2{\cdot}2 \times 10^{-11}$ M) to give approximately one-half saturation of operator with repressor. BB buffer was used but the KCl concentration was varied to give the desired ionic strength. In one case ($I^{\frac{1}{2}} = 0{\cdot}13$), a one-third dilution of BB was used. Unlabeled $\lambda\phi$80*dlac* DNA was added to 16·5 μg/ml. and then at regular time intervals 0·2-ml. samples were taken and diluted to 2·1 ml. with BB. Duplicate 1-ml. vol. were then immediately filtered as usual. This procedure ensures that the ionic strength during filtering is sufficiently low that good retention of RO complexes is obtained. For each ionic strength the first-order rate constant for dissociation (k_b) was calculated from a semilog plot of the data as described in the legend of Fig. 3.

of the method. The reaction is stopped at the indicated time by the addition of excess (usually 100-fold or more) unlabeled *dlac* DNA. The formation of new ^{32}P-labeled RO complex is thus prevented within one or two seconds; but because of the slowness of the dissociation reaction, the existing complexes are not significantly affected for several minutes, giving ample time for filtration. With this modification the method is capable of excellent precision. As illustrated in Figure 5, there is a rapid increase in RO complex over the first two minutes after mixing and equilibrium is essentially reached within ten minutes. Although minutes are required to reach equilibrium, it is apparent that the association reaction is extremely rapid because very dilute solutions are being used.

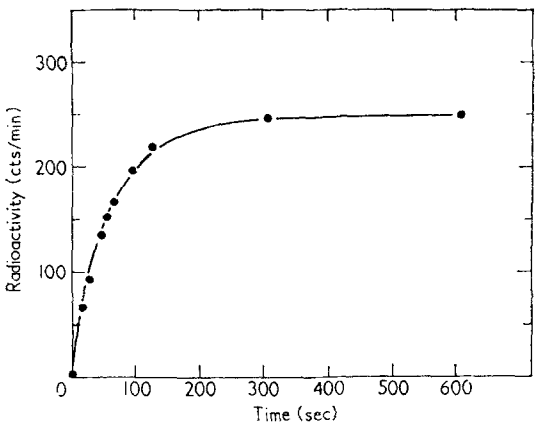

FIG. 5. Kinetics of formation of repressor–operator complex.

Using an Eppendorf pipette, 0·1 ml. of repressor solution was added to 3·0 ml. of BB buffer at room temperature containing 0·033 μg ^{32}P-labeled $\lambda\phi 80dlac$ DNA/ml. Timing was begun and the solution was quickly mixed with the pipette tip. After the desired incubation period, the reaction was stopped by adding unlabeled $\lambda\phi 80dlac$ DNA to 1·5 μg/ml., again mixing quickly with the pipette tip. Beginning precisely 1 min after adding the unlabeled DNA, triplicate 1-ml. samples were filtered as usual. This procedure was repeated for each incubation period. A background of 100 cts/min (6·5% of total) retained when the unlabeled DNA was added to the ^{32}P-labeled DNA before repressor was added has been subtracted throughout. From the DNA concentration and the molecular weight of $\lambda\phi 80$ DNA (30×10^6) the operator is easily calculated to be $1{\cdot}1 \times 10^{-12}$ M. Knowing the repressor concentration is more difficult, but by using the methods described in Riggs, Suzuki & Bourgeois, 1970*b*, it was determined to be $2{\cdot}4 \times 10^{-12}$ M.

For a bimolecular reaction, equation (2) describes the kinetics of complex formation. This equation can be rewritten as

$$\frac{d(RO)}{dt} = k_a (O - RO)(R - RO) - k_b (RO), \tag{5}$$

where O and R are respectively the molar concentration of total operator and total repressor and the other symbols are as defined previously. It will be seen that our experimental data are consistent with this equation. The quantitative determination of the second-order rate constant for association, k_a, depends, of course, on methods for independently measuring O, R, RO and to a lesser extent k_b. The determination of the first three variables has been extensively discussed in an earlier paper (Riggs *et al.* 1970*b*). We will, nevertheless, briefly review here how they are determined. The initial

operator concentration, O, is calculated from the DNA concentration by assuming one operator per phage genome, 30×10^6 daltons. The initial repressor concentration, R, is determined by titrating repressor against operator under conditions where essentially stoichiometric binding is obtained. Repressor–operator complex, RO, is calculated from membrane filtration data using a conversion factor between radioactivity retained on the filter and the radioactive complex concentration pre-existing in solution. The conversion factor is obtained by filtering a known concentration of operator saturated with repressor. Given one operator per genome, a reasonable but unproved assumption, then the concentration of R, O and RO for each experiment and time-point are known with considerable precision. The value for R is the least accurate, but even here there is almost certainly less than a 20% error (see Riggs *et al.*, 1970*b*). The rate constant for dissociation, k_b is, of course, accurately known from the experiments described in section (a) above. The determination of k_a from our kinetic experiments should, therefore, be quite accurate. The data shown in Figure 5 lead to a k_a value estimate of 7×10^9 M^{-1} sec^{-1}.

That our data are consistent with a simple bimolecular reaction characterized by this fast rate constant is well illustrated by the fact that the curve drawn in Figure 5 was not drawn through the data points, but rather was calculated from the integrated form of equation (4):

$$\ln \frac{2\,k_a\,(RO) + b - \sqrt{-q}}{2\,k_a\,(RO) + b + \sqrt{-q}} - \ln \frac{b - \sqrt{-q}}{b + \sqrt{-q}} = t \tag{6}$$

where

$$-b = k_a\,(O) + k_a\,(R) + k_b$$
$$q = 4\,k_a^2\,(R)\,(O) - b^2.$$

The experimental data obtained are clearly those which could be expected for: $O = 1 \times 10^{-12}$ M, $R = 2{\cdot}4 \times 10^{-12}$ M, $k_b = 6 \times 10^{-4}$ sec^{-1} and $k_a = 7 \times 10^9$ M^{-1} sec^{-1}.

For the initial two-thirds of the reaction the second term in equation (5) is less than 10% of the first and the familiar integrated rate equation for a bimolecular reaction will apply.

$$\frac{1}{R - O} \ln \frac{O\,(R - RO)}{R\,(O - RO)} = k\,t. \tag{7}$$

The data shown in Figure 6(a) illustrate that this is indeed the case. The line drawn in Figure 6(a) gives $k = 7{\cdot}4 \times 10^9$ M^{-1} sec^{-1}. Figure 6(a) also illustrates that experiments done on different days and at different concentrations of repressor and operator lead to essentially the same estimate for k_a. In BB buffer, k_a equals $7 \pm 0{\cdot}9 \times 10^9$ M^{-1} sec^{-1}, where the range is the standard deviation for ten experiments.

So far as has been tested, the reaction is first order with respect to both repressor and operator. It should be pointed out, however, that due to experimental limitations the reactants have been varied only over a fivefold range. The experimental limitations result from the fact that with repressor in excess, sufficient counts were not obtained when R was less than 10^{-12} M. On the other hand, when R was greater than about 5×10^{-12} M the reaction was so fast that accurate data were difficult to obtain in the early portion of the reaction. Nevertheless, over the range tested, the results are consistent with a simple bimolecular reaction between repressor and operator.

Several experiments were done with operator in excess of repressor. This is desirable since the operator concentration is easily and directly determined and the value for the

component in excess predominates in the calculation of k_a. One such experiment is shown in Figure 6(b). However, technical limitations again have made it difficult to obtain accurate data. About 5% of the DNA is retained on membrane filters even in the absence of repressor. We subtract this background to obtain the specific retention. With operator in excess, this background is high relative to the specific counts and imprecise data result. Nevertheless, as shown in Figure 6(b) the data obtained indicate that k_a is 5 to 10×10^9 M^{-1} sec^{-1}, thus confirming the previous estimate.

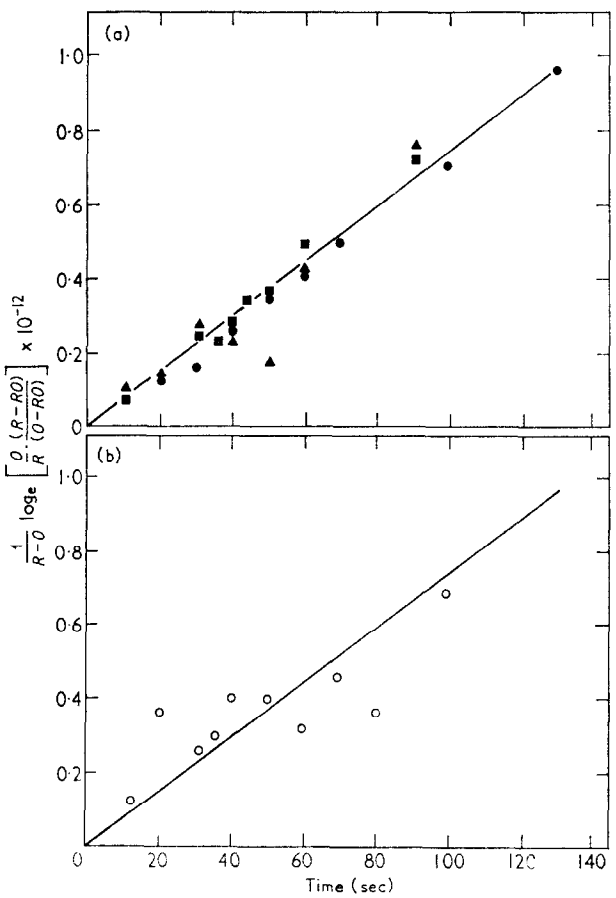

FIG. 6. Linear plots of association kinetics data.

Except for the different concentrations of repressor and operator and the presence of 0·4 μg of chicken blood DNA/ml. to eliminate any binding not due to repressor, the experiments were performed as in the legend of Fig. 5. At each time-point the concentration of RO in solution was calculated using a conversion factor obtained by filtering a known amount of RO complex under the same experimental conditions (100-fold excess of unlabeled $\lambda\phi 80dlac$, etc). The data are plotted as is customary for a second-order reaction (see text).

(a) Repressor in excess: —●—●—, $1{\cdot}0 \times 10^{-12}$ M operator and $2{\cdot}4 \times 10^{-12}$ M repressor; —▲—▲—, $0{\cdot}57 \times 10^{-12}$ M operator and $1{\cdot}2 \times 10^{-12}$ M repressor; —■—■—, $0{\cdot}67 \times 10^{-12}$ M operator and $2{\cdot}4 \times 10^{-12}$ M repressor.

(b) Operator in excess: $1{\cdot}0 \times 10^{-12}$ M repressor and $2{\cdot}1 \times 10^{-12}$ M operator.

(d) *Effect of reaction conditions on* k_a

(i) *Temperature*

The rate of association as a function of temperature is shown as an Arrhenius plot in Figure 7. The reaction is relatively insensitive to temperature; however, significant changes were measured, the reaction being about four times slower at 1°C than at 24°C. From Figure 7, we calculate that the Arrhenius activation energy for the association reaction is around 8·5 kcal/mole.

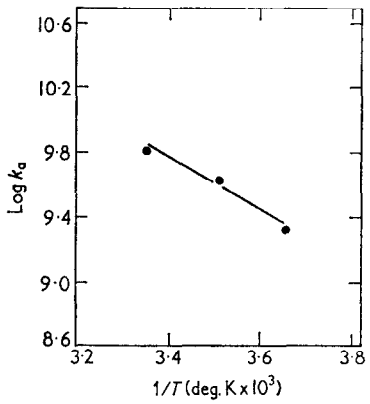

FIG. 7. Arrhenius plot of the association rate constant (k_a) as a function of temperature. Complete experiments were done similar to those shown in Fig. 6, each being at a different temperature. Since Tris buffer changes pH with temperature, care was taken to adjust each BB buffer solution to pH 7·4 at the desired temperatures. For each experiment, the rate of association (k_a) was calculated from the slope of a linear plot of the data.

(ii) *Ionic strength*

The rate of association is quite sensitive to ionic strength, becoming slower as the salt concentration is increased. The rate is about 100 times slower in BB buffer containing 0·1 M-KCl than it is in BB buffer alone. The effect of KCl concentration is illustrated in Figure 8, where the logarithm of k_a is plotted against the square root of the ionic strength. This sensitivity to ionic strength strongly suggests that the binding of repressor to operator is aided by electrostatic attraction between the negatively charged DNA chain and positively charged groups on the repressor. This point will be considered more fully in the Discussion.

(iii) *pH*

The effect of pH on the rate of association is shown in Figure 9. Only the pH range 7 to 9 has been studied; however, in this range, pH has a small but significant effect. Between pH 7 and 7·5 only a slight effect is seen; then between 7·5 and 8·2 the rate decreases about threefold. Increasing the pH above 8·3 causes little additional change.

(e) *Comparison of* k_a, k_b *and* K

The equilibrium dissociation constant, K, for the repressor–operator interaction has been determined directly under a variety of experimental conditions by measuring the equilibrium concentration of RO complex as a function of repressor (or operator) concentration (Riggs *et al.*, 1970*b*). If the steps that are rate limiting under equilibrium

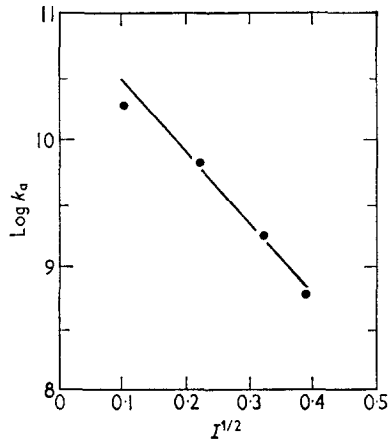

FIG. 8. Effect of ionic strength on the association rate constant (k_a).
Complete experiments were done similar to those shown in Fig. 6, each being at a different ionic strength. In general, BB buffer was adjusted to the desired ionic strength by adding KCl. For experiments requiring an ionic strength less than our standard buffer, the Tris and magnesium acetate components of BB buffer were reduced to 5 and 2 mM, respectively. For each experiment a linear plot of the data was made and k_a calculated from the slope.

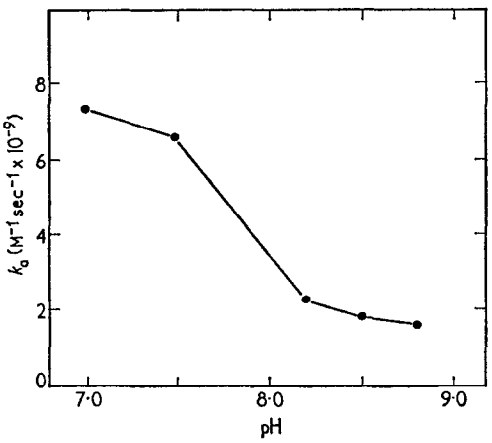

FIG. 9. Effect of pH on the association rate constant (k_a).
Complete experiments were done similar to those shown in Fig. 6, each being at a different pH. The stock repressor solution was kept in standard BB and diluted into the reaction solution at the desired pH. The final pH was then measured. For each experiment, k_a was calculated from the slope of a linear plot of the data.

conditions are also rate limiting for the kinetic experiments, then the ratio of k_b to k_a should equal K. We do, in fact, find excellent agreement between K and k_b/k_a. This is well illustrated in Figure 10. The data points in this Figure are equilibrium constants measured directly at several different ionic strengths and are reproduced from Riggs *et al.* (1970*b*). The dashed line is that which was obtained from the data on the rate of dissociation shown in Figure 4, and the rate of association shown in Figure 8. The difference between K and k_b/k_a is small throughout the entire range of ionic strength.

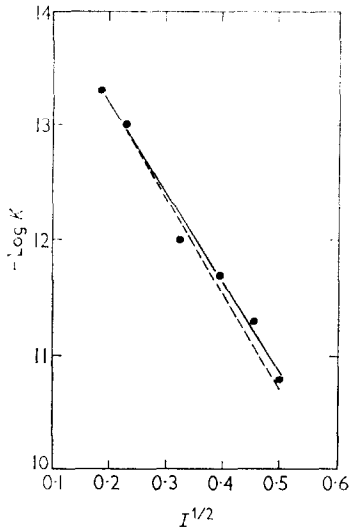

FIG. 10. Comparison of equilibrium constants measured directly and those calculated from the ratio k_b/k_a.

Equilibrium constants (K) have been previously measured at several different ionic strengths by doing binding curves under equilibrium conditions (Riggs, Suzuki & Bourgeois, 1970*b*). These data are reproduced here so that a comparison can be made with the equilibrium constants calculated from the kinetic data reported here.

—●—●—, Direct measurement of K. ----, the ratio k_b/k_a calculated using the lines drawn through the data points shown in Figs 4 and 8.

Our kinetic experiments indicate that the binding of repressor to operator should become weaker by a factor of three on going from pH 7 to 8·5. We did not detect this small change with our equilibrium measurements, but this is not surprising because equilibrium experiments would have to be done very carefully to detect this small change. For this reason, we also did not obtain precise information about the effect of temperature from equilibrium measurements, but for the most carefully done experiments a slight weakening of the binding at 1°C was detected. The results obtained from our kinetic experiments confirm the equilibrium results and are more precise. Small changes in the kinetic constants are easier to detect than small changes in the equilibrium constant. From the kinetic data shown in Figures 3 and 7 the binding is less tight by a factor of four at 1°C than it is at 24°C.

From the change in equilibrium constant with temperature, ΔH for the binding reaction is $+ 8{\cdot}5$ kcal. mole^{-1}. At 24°C the equilibrium association constant is 1×10^{13} M^{-1}; therefore, ΔF for the reaction is -18 kcal. mole^{-1}. The entropy change, ΔS, is thus $+ 90$ cal. mole^{-1} deg.$^{-1}$, and is clearly the main driving force for the reaction. This change in entropy could arise from a change in configuration of repressor and/or operator. But, as is more likely, it could result from changes in the solvation of the reacting species.

4. Discussion

An extremely important question that remains to be answered is: How does a protein specifically recognize a unique sequence of bases in duplex DNA? What is the nature of the binding? The problems introduced by these questions can now be attacked with powerful tools since we report here that both the rate of dissociation (k_b) and the rate

of association of repressor and operator (k_a) can be directly and accurately measured by simple experiments. These kinetic constants are what, in the final analysis, determine the repressor–operator equilibrium. A detailed analysis of this fundamental protein–DNA interaction will aid future *in vitro* work and should also aid in understanding the *in vivo* expression of the *lac* operon. It will be seen later in the discussion that our initial kinetic studies reported here already have important implications as to the molecular mechanism of binding.

(i) *Rate of dissociation*

In order for the repressor to recognize the *lac* operator as unique in the *E. coli* genome, it probably interacts with at least 12 bases. Even this number of very weak bonds would be expected to result in very tight binding, so it is not surprising that the rate of dissociation of repressor and operator is very slow, with a half-life of 19 minutes in our standard buffer (0·05 M ionic strength). However, the rate of dissociation is sensitive to ionic strength. Extrapolation of the data in Figure 4 to zero ionic strength gives a half-life as long as 60 minutes. On the other hand, at an ionic strength of 0·2, which may be closer to physiological conditions, the half-life for dissociation is only five to six minutes. *In vivo*, the *lac* operon can be fully induced in less than one minute because inducers bind to the RO complex and greatly accelerate the rate of dissociation (Riggs *et al.*, 1970*a*). The rate of dissociation is not sensitive to pH in the range 7 to 9 nor is it sensitive to temperature. This latter result is somewhat surprising but indicates that the Arrhenius activation energy for dissociation is close to zero. The enthalpy content of stable and "activated" RO complexes must be nearly the same.

(ii) *Rate of association*

The rate of association of R and O is very fast. Kinetics appropriate for a simple bimolecular reaction are followed with $k_a = 7 \times 10^9$ M^{-1} sec^{-1} in our standard buffer.

The association of uncharged macromolecules would be expected to be limited by diffusion to a slower rate than this. The diffusion rate limit is usually estimated from v. Smoluchowski's equation (v. Smoluchowski, 1917; Alberty & Hammes, 1958):

$$k_a = \frac{4\pi N}{1000} r_{12} D_{12} \tag{8}$$

where r_{12} is the reaction radius, D_{12} is the sum of the diffusion coefficients for the reactant species and N is Avogadro's number. This equation assumes that every collision is productive; thus it gives a maximum estimate. The *lac* repressor should have a diffusion coefficient of about 5×10^{-7} cm^2 sec^{-1} on the basis of its 150,000 molecular weight. The diffusion coefficient for the operator is not known, but can hardly be greater than that of the repressor since operator is in 30×10^6 molecular weight DNA. The reaction radius for most reactions is about 5×10^{-8} cm, so one would estimate that the maximum rate constant for RO formation should be on the order of 10^8 M^{-1} sec^{-1}. The value we find for k_a is thus more than one order of magnitude greater than that expected for a diffusion-controlled reaction.

We have carefully examined our procedures for sources of error leading to underestimation of either operator or repressor concentration and none was found. Possible errors in the determination of the reactant concentrations have been extensively analyzed and discussed in a previous paper (Riggs *et al.*, 1970*b*). Perhaps the most convincing argument that the methods for determining repressor and operator concentration are valid is that before extensive repressor purification, one operator

binding site is found for every four inducer (isopropyl-β-D-thiogalactoside) binding sites, a very reasonable result since the repressor is a tetramer. In addition, experiments with these unpurified repressor preparations confirm the rate constants reported here (Jobe, Riggs & Bourgeois, manuscript in preparation).

The reason for the extremely fast reaction rate is almost certainly the fact that there is electrostatic attraction between a positively charged site on the repressor and the negatively charged phosphate groups in the operator. There are two primary reasons to think that electrostatic forces are important in the binding: (1) the sensitivity of repressor–operator binding to ionic strength; (2) the repressor binds to and can be purified on phosphocellulose and DNA cellulose, even at pH 7, where the repressor has a net negative charge (Riggs & Bourgeois, 1968; unpublished results). It will be seen that such relatively long-range attractive forces between repressor and DNA would be expected to accelerate greatly the association reaction over that predicted by v. Smoluchowski's equation.

As far as we are aware, there is no adequate theoretical treatment of the rate of association between electrically charged large macromolecules. Therefore, an accurate quantitative analysis of our results in terms of an established theory is precluded. Perhaps the experimental data and methods reported here will aid in the development of this field of physical chemistry. Alberty & Hammes (1958) have, however, considered the binding of a charged low molecular weight substrate to a site on an enzyme and derived an equation to describe the rate of association as a function of ionic strength:

$$\log k = \log k_0 + z_1 z_2 I^{\frac{1}{2}} \tag{9}$$

where z_1 and z_2 are the charges of the substrate and the active site on the enzyme. Equation (9) is of the same form as the Brønsted–Debye–Hückel equation used to describe reactions between simple ions, so one of the reactants being a macromolecule need not alter the response of the reaction to ionic strength. Even though this equation cannot be considered theoretically sound at ionic strengths above the limiting law region, the relationship between substrate binding rates and ionic strength (or K_M and ionic strength) has been found to be of this form even at the high ionic strengths (0·01 to 0·2 M) used for our work (Alberty & Hammes, 1958; Hammes & Alberty, 1959). We find that the relationship between ionic strength and k_a for RO formation is in agreement with equation (9). The slope of the line in Figure 8 is 10, a reasonable value for $z_1 z_2$, since the operator contains at least this many phosphate groups. Even though any analysis based on equations derived for enzyme–substrate binding can be only qualitative at best, it nevertheless seems clear from the theoretical analysis of Alberty & Hammes that electrostatic attraction between repressor and operator would be expected to accelerate greatly the rate of association.

The rate we measure should not, therefore, be considered "impossible". However, even after taking the acceleration due to electrostatic interaction into account, the reaction remains very fast and the rate of association of repressor and operator is probably diffusion-limited. In support of this statement is the fact that in 20% sucrose the rate is reduced by a factor of two, as would be expected from the change in viscosity.

It seems certain that the repressor is not simply diffusing randomly but rather is oriented by relatively long-range electrostatic forces toward DNA. It is therefore worth considering an extreme model of oriented diffusion. This model is that the *lac* repressor searches for the operator not by performing a three-dimensional random walk,

but rather by binding to DNA and "rolling" or "hopping" along it, thus reducing the search for operator to only two dimensions. Several arguments can be brought against this interesting idea, however. First, the non-specific affinity of the *lac* repressor for non-operator DNA is very weak, being characterized by a dissociation constant not less than 10^{-3} M, based on phosphate concentration (Riggs *et al.*, 1970*b*, and unpublished data). Second, such extreme orientation is unnecessary to explain the rapidity of repressor–operator binding. As indicated in the above paragraph, electrostatic attraction would be expected to accelerate the binding rate even if the operator were not in a long chain of DNA. Third, if rolling along the DNA were a significant factor, then fragmenting the DNA to small pieces should reduce the rate of association (and change the equilibrium constant), and an effect of this kind has not been detected. The operator in sonicated $\lambda\phi 80dlac$ DNA (mol. wt $\simeq 1\times10^6$) competes just as well with ^{32}P-labeled $\lambda\phi 80dlac$ DNA as unsonicated DNA (Bourgeois & Riggs, 1970). This means that the repressor–operator equilibrium constant for sonicated DNA is the same as for intact DNA; therefore, k_a seems certain to have stayed the same. We also have preliminary evidence that operator in $\lambda\phi 80dlac$ DNA degraded to fragments of less than 60,000 molecular weight retains good competing activity. Thus the rolling model is not supported by the present data. Future work with even smaller operator fragments is called for, however.

(iii) *Equilibrium constant*

The kinetic experiments lead to the same estimate for the equilibrium constant as did our earlier equilibrium studies. The ratio k_b/k_a agrees very well with the experimentally determined K over a wide range of salt concentration. This remarkable agreement between k_b/k_a and K can hardly be fortuitous. It is probably the strongest evidence that our kinetic experiments are giving true rate constants. The agreement also means that the steps in the binding reaction that are rate-limiting for the kinetic experiments are also rate-limiting under equilibrium conditions. The rate constants can generally be more accurately determined than can the equilibrium constant, so small differences (a factor of two or less) become meaningful.

There is about a threefold increase in k_b/k_a between pH 7 and 8·5, indicating that the binding becomes less tight as additional negative charges are added to the repressor. This rather small effect results from a decrease in the rate of association. Since the magnitude of the effect is so small, whatever group is being titrated need not be part of the active site (see Hammes & Alberty, 1959).

The effect of temperature on k_b/k_a is such that the binding is less tight at 1 than at 24°C by about a factor of four, again the change being confined to the rate of association. This result confirms our earlier equilibrium studies (Riggs *et al.*, 1970*b*). In our standard buffer at 24°C the equilibrium constant is 1×10^{13} M^{-1}, corresponding to a ΔF equal to -18 kcal. mole^{-1}. From the change in equilibrium constant with temperature, ΔH equals about $+8{\cdot}5$ kcal. mole^{-1}. Therefore, in terms of heat energy, the binding of repressor to operator is actually unfavorable. The driving force for the reaction comes from the entropy change which equals $+90$ cal. mole^{-1} deg.$^{-1}$ at 24°C. This large entropy change could result from configurational changes in the repressor or operator and/or the freeing of water molecules on binding. DNA has been postulated to be surrounded by a shell of structured water molecules (Crothers, 1964); perhaps the repressor disrupts this shell. It may or may not be of significance that the binding of actinomycin D to DNA is also governed primarily by entropy changes

(Müller & Crothers, 1968). It is interesting to note that since the driving force for the binding reaction is the entropy change rather than specific bond formation between the repressor and the bases in DNA, it is quite possible that specificity results only from a lack of steric hindrance, given the correct base sequence.

(iv) *Binding mechanisms*

Gierer (1966) has proposed what seemed to be a very attractive model for specific protein–DNA interaction. He postulated that the sequence in the operator region was such (reverse complementary repeat) that the operator could exist in two reasonably stable forms: the normal duplex structure, and a branched structure stabilized by intrastrand complementary base pairing. This unique transfer RNA-like structure could, of course, readily be recognized by regulatory proteins. Our results suggest that the simple form of the Gierer model does not apply for the *lac* system. The branched structure would be expected to have at least ten unpaired bases and would, therefore, be less stable than the normal duplex form. We have shown that reassociation of denatured DNA at 60°C leads to complete restoration of operator activity (Riggs *et al.*, 1970*b*). The operator is thus in a thermodynamically stable form. The linear and branched form of the operator could be in equilibrium but the linear form should greatly predominate. Furthermore, one would expect that at room temperature the transition between the linear and branched form would be slow because several bonds need to be broken. Because the association of repressor and operator has been found to be so fast, it hardly seems likely that only a small fraction of the operators are available for binding at any given moment. If this were the case, most collisions would be ineffective and this seems not to be allowed. The above considerations mediate against any model which requires that the operator must first spontaneously open up or "breathe" before repressor binding. Therefore, the Gierer model seems extremely unlikely, *unless* the *lac* operator contains unusual bases that cannot participate in normal hydrogen bonding and stacking.

Another possible binding mechanism is that the repressor destabilizes the operator region and causes the operator to open up, thereby exposing the bases. In this model there is first non-specific binding followed by specific binding. The repressor does bind non-specifically, but note that it is the rate of specific binding that we measure. For every effective encounter, the *lac* repressor must have made countless other encounters where the DNA was destabilized, but because the sequence was wrong, dissociation quickly followed. The weak non-specific binding observed for the *lac* repressor is probably not sufficient to cause such destabilization of the helical structure and, of course, it is difficult to reconcile the extreme speed of binding with this model. This line of thinking, however, does point out that it is important to determine the stability of DNA in the presence of a high concentration of repressor.

The final model that should be considered is that the repressor merely binds to the outside of Watson–Crick duplex DNA and reads the edges of the bases exposed in the large and/or small grooves. This is the model which we strongly favor at present because of: (a) simplicity, (b) compatibility with our kinetic data, and (c) the requirement for double-stranded DNA (Riggs *et al.*, 1970*b*). The shape and hydrogen bonding potentiality of the exposed edge of an AT pair is quite different from that of a GC pair, and since the repressor looks at the DNA from a fixed orientation, there are four different base pairs. If the repressor can fit into the grooves, specificity of binding would easily follow.

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