Equilibrium binding of EcoRI endonuclease to DNA has been analyzed by nitrocellulose filter and preferential DNA cleavage methods. Association constants for pBR322 and a 34-base pair molecule containing the EcoRI site of this plasmid in a central position were determined to be $1.9 \times 10^{11}$ M$^{-1}$ and $1.0 \times 10^{11}$ M$^{-1}$ at $37^\circ C$, respectively, with the stoichiometry of binding being $0.8 \pm 0.1$ mol of endonuclease dimer per mol of DNA. In contrast, the affinity of the enzyme for a pBR322 derivative from which the EcoRI site has been deleted is $3.2 \times 10^{8}$ M$^{-1}$ as judged by competitive binding experiments. If it is assumed that each base pair can define the beginning of a nonspecific binding site, this value corresponds to an affinity for nonspecific sites of $7.4 \times 10^{6}$ M$^{-1}$. Furthermore, the affinity of the endonuclease for the EcoRI-methylated sequence is at least three orders of magnitude less than that for the unmodified recognition site.

The dependence on temperature and ionic strength of the equilibrium constant governing specific interactions has also been examined. The temperature dependence of the reaction indicates that entropy increase accounts for 70% of the free energy of specific binding at $37^\circ C$. Affinity of the endonuclease for the EcoRI site is highly dependent on NaCl concentration. Analysis of this dependence according to the theory of Record and colleagues (Record, T. M., Jr., Lohman, T. M., and deLaseth, P. (1976) J. Mol. Biol. 107, 145–158) has implicated 8 ion pairs in the stability of specific complexes, a value identical with the number of phosphate contacts determined by ethylation interference experiments (Lu, A. L., Jack, W. E., and Modrich, P. (1981) J. Biol. Chem. 256, 13200–13206). Extrapolation to 1 n NaCl suggests that nonelectrostatic interactions account for 40% of the free energy change associated with specific complex formation.

The most widely studied of the Type II restriction endonucleases has been that of EcoRI specificity (1). The recognition sequence

$$5^{\prime}-G-A-A-T-T-C$$

$$3^{\prime}-C-T-T-A-A-G$$

exhibits 2-fold rotational symmetry with the sites of strand cleavage being symmetrically disposed about the dyad axis (2, 3). This endonuclease has been purified to homogeneity (4, 5) and the primary sequence has been determined (6, 7). The protein exists in solution as an equilibrium mixture of dimers and tetramers (5, 8). It has been argued that the dimer represents the functional unit of the enzyme and that specific endonuclease-DNA complexes may possess elements of 2-fold symmetry (1, 9). This idea has been supported by results of alkylation protection and interference experiments (10).

Although EcoRI sites are typically embedded within a large background of nonspecific DNA, the endonuclease is capable of efficient location of its recognition sequence. Several models have been proposed to explain facile site location by sequence-specific proteins (11–13). Such models invoke nonspecific binding of protein to DNA and then translocation in a dimension-limited diffusion process until the recognition site is located. As a prerequisite, such proteins must exhibit an affinity for nonspecific sequences sufficient to permit a significant level of interaction but not so high as to represent a kinetic barrier to translocation.

Several studies have examined interaction of EcoRI endonuclease with specific and nonspecific DNA sequences (9, 14–18). However, interpretation of these experiments has been complicated by substantial variation in reported binding constants. The majority of this work has relied on the nitrocellulose assay for trapping of protein-DNA complexes, but questions of membrane retention efficiency of specific and nonspecific complexes have not been addressed. Furthermore, these studies have been performed under a variety of conditions of temperature and ionic strength rendering comparison difficult, and no attempts have been made to correct apparent specific binding constants for enzyme bound to nonspecific DNA sequences (1, 13). For these reasons we have performed a systematic analysis of interaction between EcoRI endonuclease and specific and nonspecific sequences of plasmid pBR322 using the intact plasmid, a derivative from which the EcoRI site has been deleted, and a 34-base pair DNA which contains the EcoRI site of the molecule. In these studies we have employed two assays for specific binding, and further, have examined the dependence of specific interactions on temperature and ionic strength.

**Experimental Procedures**

**Materials**

EcoRI endonuclease and methylase were homogeneous preparations isolated as previously described (5, 19). Plasmid pBR322 DNA (20) and pBR322ΔRI DNA (17) were isolated according to Hardies and Wells (21). T4 polynucleotide kinase was a gift from Dr. R. Kolodner (Harvard University). Escherichia coli alkaline phosphatase and PolII endonuclease were purchased from commercial sources.

$[\gamma^{32P}]ATP$ was prepared by the method of Glynn and Chappell (22). RPC-5 resin was provided by Dr. G. D. Novelli (Oak Ridge National Laboratory). Nitrocellulose BA-85 membranes were purchased from Schleicher & Schuell."
**Methods**

DNA Substrates—Plasmid pBR322 (4362 bp') was hydrolyzed with PvuII endonuclease to yield a molecule with the EcoRI endonuclease site near the middle (2067 bp from the nearest end). A 34-bp fragment containing the EcoRI site of this plasmid in a central position (16 bp from the nearest end) was prepared as previously described (10). DNA fragments were 5'-end labeled with T4 polynucleotide kinase (22) and purified by reverse phase system 5 chromatography (24).

Plasmid pBR322.RI is a derivative of pBR322 that has been cleaved with EcoRI endonuclease, DNA termini repaired with T4 DNA polymerase, and then circularized using T4 DNA ligase (17). Linear pBR322.RI was obtained by PvuII endonuclease hydrolysis.

Plasmid pBR322 and 34-bp fragment were methylated with EcoRI methylase in vitro as previously described (19). The extents of methylation were 1.8 and 1.9 methyl groups incorporated per DNA molecule, respectively.

Assays of DNA Binding—Specific endonuclease-DNA complexes formed in the absence of Mg\(^{2+}\) are efficiently retained on nitrocellulose filters (5, 14, 17, 18). Unless specified otherwise, reaction mixtures contained 0.1 M Tris-HCl, pH 7.6, 1 mM EDTA, 0.05 mg/ml of bovine serum albumin, [\(^{32}\)P]DNA, and EcoRI endonuclease as indicated. Incubation was at 37 °C until well after equilibrium was attained, usually 1-2 h. Duplicate reaction samples were filtered through nitrocellulose membranes (presoaked in 0.1 M Tris-HCl, pH 7.6, 1 mM EDTA) and the DNA retained was quantitated by liquid scintillation counting. Analysis of the temperature dependence of DNA binding was performed in a similar manner. However, the pH was adjusted to 7.6 at the incubation temperature in order to compensate for the temperature dependence of the Tris buffer.

An alternate method was also employed for assay of specific EcoRI endonuclease-DNA complexes (25). In this procedure enzyme and [\(^{32}\)P]DNA are allowed to reach equilibrium with respect to specific complex formation at 37 °C in the absence of Mg\(^{2+}\) as described above. Mg\(^{2+}\) and a large excess of the same DNA in nonradioactive form is then added, incubation continued for 20 s, and the reaction quenched. Under these conditions the fraction of labeled DNA cleaved is directly proportional to the amount of enzyme-DNA complex formed at equilibrium with the yield being 0.8-1.0 mol of double strand breaks per mol of endonuclease dimer at saturating DNA concentrations.

Analysis of ionic strength dependence of specific binding of EcoRI endonuclease to pBR322 was determined in reactions containing 0.02 M Tris-HCl (pH 7.6), 1 mM EDTA, 0.08 mg/ml of bovine serum albumin, 0.05-0.25 M NaCl, and DNA and endonuclease as indicated. Reactions were incubated at 37 °C until equilibrium was attained (empirically determined) before analysis by nitrocellulose membrane filtration.

DNA concentrations were determined by two methods. The concentration of stock solutions of pBR322 and derivatives of pBR322 were determined spectrophotometrically using an extinction coefficient of 20 liters/g cm at 260 nm and a molecular weight of 2.88 × 10\(^6\) g/mol (26). The concentration of 34-bp [\(^{32}\)P]DNA (above) was determined by isotope dilution using a preparation of the unlabeled molecule whose concentration had been accurately determined spectrophotometrically. DNA concentrations are expressed in terms of molecules unless otherwise stated. EcoRI endonuclease concentrations were determined using an extinction coefficient of 0.830 liter/g cm at 278 nm and are expressed in terms of dimer equivalents (5).

**RESULTS**

Specific Binding—EcoRI endonuclease-DNA complexes are efficiently retained on nitrocellulose membranes in the absence of Mg\(^{2+}\) (5, 14, 17, 18). Incubation of a fixed concentration of pBR322 or a 34-bp DNA containing the EcoRI site of this plasmid with increasing endonuclease results in hyperbolic binding isotherms that appear to represent a true equilibrium process (Fig. 1). EcoRI endonuclease titration of pBR322 occurred with an apparent stoichiometry of 0.78 ± 0.08 mol of endonuclease (dimer) per mol of pBR322 and an association constant of 1.9 ± 0.6 × 10\(^{10}\) M\(^{-1}\) at 37 °C. The 34-bp molecule behaved similarly (Fig. 1) with an association constant of 1.0 ± 0.4 × 10\(^{11}\) M\(^{-1}\) and a stoichiometry of 1.0 ± 0.2 mol of enzyme per mol of DNA at 37 °C. The converse titration of a fixed endonuclease concentration with pBR322 DNA yielded specific association constant \(K_S = 1.7 \times 10^3\) M\(^{-1}\) and a stoichiometry of 0.93 mol of pBR322 bound per mol of endonuclease (Fig. 2). A number of experiments in which endonuclease was incubated with near saturating (>65%) DNA concentrations yielded a stoichiometry of 0.8 ± 0.1 mol of endonuclease dimer per mol of EcoRI site. This value indicates that at least 80% of the enzyme was active in specific binding and that efficiency of retention of specific complexes on nitrocellulose membranes is near unity. In the experiments reported here we have assumed that this stoichiometry represents the fraction of active enzyme, rather than a filter retention effect, and have incorporated this correction.

The abbreviation used is: bp, base pair.

1 W. E. Jack and P. Modrich, manuscript in preparation.
into analysis of binding curves. During the course of analysis of specific interaction between the endonuclease and DNA, all binding curves found to be hyperbolic indicating the absence of cooperative effects in specific binding. Since EcoRI endonuclease also interacts with non-specific DNA sequences (Refs. 14, 15, and below) the observed association constant is expected to be a function of the DNA concentration (13),

$$K_{obs} = K_s/(1 + |D|K_s)$$

where $K_s$ is the intrinsic specific association constant, $K_N$ is the non-specific association constant, and $D$ is the free DNA concentration (in base pair equivalents), which in the case of experiments presented here can be approximated by the total DNA concentration. Using the non-specific association constants described below, it can be shown that under the experimental conditions of Fig. 1, $K_{obs} = K_s/(1 + 0.03)$ the case of pBR322 and $K_{obs} = K_s/(1 + 0.002)$ for the 34-bp DNA. Hence, under the dilute conditions of these experiments the difference between observed and intrinsic association constants is well within experimental uncertainty. Furthermore, no variation in the experimentally determined association constant was observed over a 100-fold range of endonuclease and 6-fold range of DNA concentration. Given the similar specific association constants for pBR322 and the 34-bp molecule derived from this plasmid, these experiments also illustrate that the affinity of the endonuclease for the EcoRI site of the plasmid is much greater than that for the remainder of the 4362-bp molecule.

One possible reservation concerning the results cited above is the potential for perturbation of DNA-protein complexes during collection on nitrocellulose membranes. Therefore, formation of specific complexes was monitored by an alternate method in which pre-existing DNA-endonuclease complexes were scored as those susceptible to preferential cleavage upon addition of Mg$^{2+}$ (25). Using this assay, we have demonstrated that binding isotherms are independent of method of measurement. Fig. 3 presents results of an experiment in which specific binding was scored by both methods. As can be seen, the two procedures yield comparable results. In the case of the preferential cleavage assay, the binding curve shown yields an association constant for binding to pBR322 of $1.8 \times 10^{10}$ M$^{-1}$, a value identical with that cited above.

![Graph](https://example.com/graph.png)

**Fig. 3.** Comparison of nitrocellulose filter and DNA cleavage assays for quantitation of specific DNA-endonuclease-DNA complexes. [$^{32}$P]pBR322 (1.27 pm) and EcoRI endonuclease (dimer) as indicated were incubated at 37°C for 1 h. Samples (0.5 ml) were removed and analyzed by the nitrocellulose filter binding assay (1). The remainder was supplemented with unlabeled pBR322 and MgCl$_2$ to yield final concentrations of 5.4 nM and 9 nM, respectively. After a 20-s incubation at 37°C, the reactions were quenched and subjected to aqueous electrophoresis (25). Radioactive bands were excised and quantitated by liquid scintillation counting (A).

$\text{Table I}$

**Association constants for the interaction of EcoRI endonuclease with DNA**

| Substrate       | $K$ (M$^{-1}$) |
|-----------------|---------------|
| pBR322          | 1.9 ± 0.6 x 10$^{11}$ |
| 34-bp fragment  | 1.0 ± 0.4 x 10$^{11}$ |
| Me$_2$-pBR322   | 3.2 ± 0.7 x 10$^{10}$ |
| Me$_2$-34-bp fragment | 2.4 ± 0.1 x 10$^{10}$ |
| pBR322ARI        | 1.9 ± 0.5 x 10$^8$ |

Experiments were performed at 37°C as described under "Methods." The equilibrium constants are expressed in terms of DNA molecules.

![Graph](https://example.com/graph.png)

**Fig. 4.** Binding of EcoRI endonuclease to EcoRI modified and non-specific DNA sequences as monitored by competition methods. Linear pBR322ARI (O) or covalently closed circular pBR322ΔRI (C) was incubated with 9.70 pm [$^{32}$P]pBR322 and 7.34 pm EcoRI endonuclease at 37°C (see under "Methods"). EcoRI methylated pBR322 (A) was incubated with 9.59 pm [$^{32}$P]pBR322 and 4.94 pm endonuclease. Specific complexes were quantitated by the nitrocellulose filter assay. Extrapolation of the Dixon plot yields an intercept on the abscissa equal to $-K_s/(1 + [S]/K_s)$ where $K_s$ and $K_N$ are dissociation constants (27). The data was fit by a linear least squares regression analysis. The inhibition constants were determined to be $3.1 \times 10^{-17}$ m and $4.1 \times 10^{-17}$ m for pBR322ARI and EcoRI methylated pBR322, respectively. The corresponding association constants are $3.2 \times 10^{10}$ M$^{-1}$ for pBR322ARI and $2.4 \times 10^{10}$ M$^{-1}$ for EcoRI methylated pBR322.

Association constants measured in this work are summarized in Table I.

**Nonspecific Binding of EcoRI Endonuclease to DNA**—Non-specific DNA-EcoRI endonuclease complexes are poorly retained on nitrocellulose membranes (17), with retention efficiencies for such complexes varying between 10 and 30% depending on the batch of nitrocellulose membranes used. We have, therefore, used competition methods to analyze nonspecific binding. In order to assess the affinity of endonuclease for pBR322 sequences external to the EcoRI site, competition studies were performed using pBR322ΔRI, a plasmid in which the EcoRI site has been destroyed by a 4-bp insertion (17). As shown in Fig. 4, specific binding of EcoRI endonuclease to pBR322 DNA was inhibited by pBR322ARI, and double reciprocal plots indicated this inhibition to be competitive in nature (not shown). In addition, covalently closed circular and linear pBR322ARI were equally effective in this respect indicating that DNA topology does not play a significant role in equilibria governing nonspecific interactions. The competition curves shown in Fig. 4 indicate that interaction of the endonuclease with sequences external to the EcoRI site of pBR322 are governed by an association constant of $3.2 \times 10^{10}$ M$^{-1}$, a value 65 times less than that for...
the EcoRI site. If it is assumed that each nucleotide pair can initiate a potential nonspecific binding site, this value corresponds to a nonspecific association constant on a per site basis of 7.4 \times 10^9 \text{ M}^{-1} under standard assay conditions (see under "Methods"). Therefore, this analysis, like that described above, indicates that affinity of the endonuclease for the EcoRI site of pBR322 is much greater than that for the remainder of the plasmid DNA molecule.

Effects of the EcoRI Modification on Binding of the Endonuclease and Its Recognition Site—Modification of the EcoRI site involves methylation of the 6-NH_2 of adenine residues adjacent to the recognition site dyad, rendering the sequence resistant to cleavage by the restriction endonuclease (3). The interaction between endonuclease and EcoRI-methylated DNAs was, therefore, examined. Since we have previously shown that complexes formed between the endonuclease and modified DNA are retained with low efficiency on nitrocellulose membranes (17), this analysis was performed by competition methods. Double reciprocal plots (1/[ES] versus 1/[S]) indicate methylated pBR322 is a competitive inhibitor of pBR322 binding to EcoRI endonuclease (not shown), and plots of 1/[ES] versus [I] (Fig. 4) yield an association constant of 2.4 \times 10^9 \text{ M}^{-1} (calculated in terms of DNA molecules) for interaction of the enzyme with the modified DNA. This value is comparable to that observed for binding of the endonuclease to pBR322ΔRI, indicating that the endonuclease has a markedly reduced affinity for the modified EcoRI sequence.

The association constant determined in this experiment may reflect interactions between endonuclease and the methylated EcoRI site, between the enzyme and sequences external to the recognition site, or a combination of both. In order to more carefully assess the affinity of the endonuclease for the methylated EcoRI site, competition experiments were performed in which binding of 34-bp [5'-32P]DNA was examined in the presence of the unlabeled methylated 34-bp molecule as competitor. In these experiments (not shown) competition by the methylated short DNA was governed by an association constant of 1.9 \times 10^9 \text{ M}^{-1}, although inhibition deviated from linear competitive above 7 nM, indicative of more than one DNA molecule binding to endonuclease (28) or the presence of trace amounts of substrate in the inhibitor (29). The possibility that the preparation of methylated DNA contained trace amounts of unmethylated molecules cannot be excluded. Nevertheless, this association constant demonstrates that affinity of the endonuclease for the unmethylated EcoRI is at least three orders of magnitude greater than that for the methylated sequence. If the methylated form of the 34-bp DNA contains multiple binding sites for the enzyme, then the difference in affinity will be even larger.

Enthalpy and Entropy Changes Associated with Specific Complex Formation—The binding of EcoRI endonuclease to pBR322 was investigated as a function of temperature. From the thermodynamic relationships at equilibrium it can be shown that 
\[
\frac{d(\ln K)}{d(1/T)} = -\Delta H^*/R (30) \]

Construction of a van't Hoff plot of ln K versus 1/T enables calculation of the enthalpy of binding ($\Delta H^*$) from the slope (Fig. 5). The values obtained are: 
\[
\Delta H^* = \Delta H(37^\circ C) = -4.7 \text{ kcal/mol}, \quad \Delta S = 36.2 \text{ cal/mol deg} (T\Delta S = -11.2 \text{ kcal/mol at } 37^\circ C).
\]

It is apparent that the primary driving force for specific EcoRI endonuclease binding is a large increase in entropy.

Dependence of Specific Complex Formation on NaCl Concentration—If a protein binds electrostatically to DNA, counterions are displaced from the phosphate backbone increasing the entropy of the system. Consequently, the position of equilibrium with respect to polyelectrolyte interactions can be altered by variation of counterion concentration. Record

\[
\frac{d \text{log } K_{\text{obs}}}{d \text{log } [M^+] = \frac{-\psi}{m^+}}
\]

where $\psi$ is the fraction of counterion bound per phosphate and $m^+$ is the number of ion pairs formed.

As shown in Table II, the equilibrium constant for EcoRI

**TABLE II**

| [NaCl] (M) | K (M$^{-1}$) |
|-----------|-------------|
| 0.055     | 9.3 \pm 0.9 \times 10^{10} |
| 0.088     | 1.6 \pm 0.1 \times 10^{10} |
| 0.106     | 2.0 \pm 0.5 \times 10^{10} |
| 0.108     | 5.0 \pm 0.3 \times 10^{10} |
| 0.125     | 1.3 \pm 0.1 \times 10^{10} |
| 0.141     | 6.4 \pm 1.1 \times 10^{10} |
| 0.165     | 2.4 \pm 0.4 \times 10^{10} |
| 0.238     | 9.6 \pm 1.4 \times 10^{10} |

**FIG. 5.** Dependence of the equilibrium association constant on temperature. The association constant for EcoRI endonuclease binding to [32P]pBR322 was measured as a function of temperature using the nitrocellulose filter binding assay. Conditions were as described in the legend to Fig. 1 except that pH was adjusted to pH 7.6 at each temperature. The line is a linear least squares regression fit, and error bars represent one standard deviation of quadruplicate determinations.

**FIG. 6.** Dependence of the association constant on NaCl concentration. The binding of EcoRI endonuclease to [32P]pBR322 was determined at 37°C as described (see under "Methods" and Fig. 1) except that reaction mixtures were 0.02 M Tris–HCl (pH 7.6), 1 mM EDTA, 0.05 mg/ml of bovine serum albumin, and the indicated NaCl concentration. Data shown is presented graphically in Fig. 6. The uncertainties are the standard deviation in the slope of the Eadie-Scatchard plot used to determine the association constant.
EcoRI Endonuclease DNA Binding

Endonuclease binding to pBR322 is quite dependent on NaCl concentration. Furthermore, the salt dependence of $K_0$ obeys the relationship predicted by polyelectrolyte theory over the range of 0.088–0.17 M NaCl (Fig. 6). Assuming $\psi = 0.88$ for double helical DNA (33), the slope of this plot yields a value for $m'$ of 8.1 ± 0.6 ion pairs formed during specific binding by EcoRI endonuclease. Subsequent extension of binding to 0.24 M NaCl does not alter the value of $m'$. Although the reason for deviation from theory of the dependence of $K_0$ on NaCl concentration at low counterion concentrations is not understood, a similar effect has been observed in the case of the lac repressor-operator system (34).

**DISCUSSION**

In order to assess interactions between EcoRI endonuclease and specific and nonspecific DNA sequences we have examined binding of the enzyme to plasmid pBR322, a derivative in which the EcoRI site has been destroyed, and a 34-bp DNA which contains the EcoRI site of the plasmid but lacks >99% of the nonspecific sequences. Results obtained with these three DNAs are internally consistent. Thus, the affinity of the enzyme for pBR322 is about 60 times that for the plasmid from which the recognition site has been deleted, a finding consistent with the observation that specific affinity for a 34-bp molecule containing the EcoRI site of pBR322 is essentially identical with that for the intact plasmid. These results are also in agreement with previous findings, based on equilibrium competition, that the intrinsic affinity of the endonuclease for the EcoRI site of pBR322 is independent of DNA chain length (25). Furthermore, if it is assumed that each base pair can represent the beginning of a nonspecific binding site, it is evident that the affinity of the endonuclease for the EcoRI site of pBR322 is on the order of 10^6 times greater than that for nonspecific sites within this DNA under the binding conditions routinely employed (see under "Methods").

We have previously concluded that the functional form of EcoRI endonuclease is the dimer based on simple steady state kinetic behavior of the enzyme in vivo and in vitro conditions where the dimer is the thermodynamically stable species ($K_d$ for the tetramer to dimer transition $\sim 10^{-12}$) (1, 5, 8). The binding isotherms reported here are also consistent with this idea. In particular, specific complex formation upon titration of DNA with the endonuclease under dilute conditions is governed by a simple hyperbolic function. This indicates that association of dimers to tetramers is not necessary for specific binding. The stoichiometry of binding (0.8 ± 0.1 mol of endonuclease dimer per mol of EcoRI sites) is also in accord with this view, and furthermore, indicates that efficiency of retention of specific complexes on nitrocellulose membranes is near unity at moderate salt concentrations.

The finding that the affinity of the endonuclease for EcoRI-modified pBR322 is comparable to that for a molecule in which the EcoRI sequence had been destroyed indicates that specific methylation markedly reduces the affinity of the endonuclease for its recognition site. In order to more carefully evaluate the affinity of the enzyme for the methylated sequence, the EcoRI-modified 34-bp DNA was employed. The affinity of the enzyme for this molecule was found to be three orders of magnitude less than that for an unmodified recognition site. This provides a minimum estimate of relative affinity of the endonuclease for methylated and unmethylated states of the EcoRI site. If the modified form of the short DNA contains multiple binding sites for the protein, then the difference could be substantially larger. However, this limit indicates that it is possible that residual affinity of the enzyme for the methylated sequence may be as much as 10 to 100 times that for a nonspecific site.

Temperature dependence of the specific association constant indicates that binding of EcoRI endonuclease to its recognition site is primarily entropy driven. At 37°C, increased entropy accounts for 70% of the binding free energy, although this should be viewed as an approximate value in light of the limited temperature range studied and the relatively small variation in $K_0$ with temperature.

The equilibrium constant for interaction of EcoRI endonuclease with its recognition site is highly dependent on salt concentration. We have examined this dependence according to the theory of Record and colleagues (31, 32) for interaction of polyelectrolytes. Although interpretations based on this approach can be complicated by dependence of polyelectrolyte hydration or amino acid residue pK values on salt concentration, as well as anion effects (31, 32), it is noteworthy that this analysis indicates 8.1 ± 0.6 ion pairs in specific complexes.

This is precisely the number predicted based on the number of phosphate contacts (4 phosphates/strand) in specific EcoRI endonuclease-DNA complexes as deduced by ethylation interference experiments.

Extrapolation of $K_0$ for specific complex formation from a NaCl concentration of 1 M can be employed to estimate the nonelectrostatic contribution to the free energy of binding (31). Utilizing the data of Table II and Fig. 6 we obtain a value of $\Delta G_m = -5.31$ kcal/mol at 37°C. The nonelectrostatic contribution to the free energy of specific site binding is given by

$$\Delta G_{m} = \Delta G_{m}^* - N \Delta G_{phosphate}$$

where $N$ is the number of ion pairs and $\Delta G_{m}^*$ is the standard free energy of formation of a single lysine-phosphate ionic interaction. In 1 M NaCl, $\Delta G_{m}^* = 0.2$ kcal/mol (31, 35). Therefore, $\Delta G_m = -5.3 - (8)0.2 = -6.9$ kcal/mol. This indicates that under our standard binding conditions (see under "Methods"), $K_0 = 1.9 \times 10^{11}$ about 40% (−6.9 kcal/mol)/−15.9 kcal/mol (0.43) of the total specific binding free energy is due to nonelectrostatic interactions.

The results cited above were obtained using a nitrocellulose membrane assay. We have also monitored specific complex formation by a preferential DNA cleavage assay, a more tedious method but one which is reliable for quantitation of specific complexes at ionic strengths up to at least 0.1 M. The fact that both methods yielded identical specific association constants indicates that the thermodynamic parameters governing specific interaction reported here are not assay dependent. While the nitrocellulose membrane assay can be used to accurately quantitate specific endonuclease-DNA complexes, it is inadequate for precise quantitation of nonspecific complexes between the enzyme and DNA due to the low efficiency of retention of such complexes on membranes (17). Depending on the lot of nitrocellulose membranes, we find that efficiency of retention of such complexes ranges from 10–30% (not shown). As shown here, this problem can be circumvented by monitoring nonspecific interactions by competition methods.

Previous studies using the nitrocellulose membrane assay have suggested that in the absence of Mg^2+, EcoRI endonuclease binds preferentially to its recognition site as opposed to nonspecific DNA sequences (5, 14, 17, 18). Interpretation of such experiments has been compromised by their failure to

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4 Since nonspecific binding constants were obtained by equilibrium competition methods in which competitor DNA was in large excess over endonuclease, values cited are expected to be biased toward nonspecific sequences which have a higher affinity for the enzyme than the typical nonspecific site. Hence, affinity for the EcoRI site relative to the average nonspecific sequence may be greater than that cited in the text.
establish retention efficiencies for enzyme-DNA complexes, particularly in the case of those involving nonspecific interactions. Indeed, Goppelt et al. (16) have concluded that there is no preferential interaction between the endonuclease and its recognition sequence in the absence of divalent cations. This conclusion was based on measurement of complex formation between EcoRI endonuclease and several oligonucleotides by circular dichroism spectroscopy. In particular, the affinities of the enzyme for d(G-A-A-T-T-C-C), d(G-A-A-T-T-C-C), d(T-T-A-C-A-T), and d(T-A-A-A-T-G) were found to be comparable (all being in the range of 3 to 8 x 10^6 M^-1), and salt dependence of binding indicated involvement of two ion pairs in binding to d(G-A-A-T-T-C-C) (16). These conclusions are clearly at odds with the results presented here. We attribute these differences to several effects. First, measurement of binding affinity by circular dichroism required micromolar concentrations of endonuclease and oligonucleotide, values well within the range of where nonspecific interactions become significant (Ref. 15 and this paper). Secondly, it has been shown that in specific complexes the endonuclease interacts with about 10 base pairs (10), with several of the phosphate contacts being external to the canonical recognition sequence. The 5'-OH terminated octamer employed in the circular dichroism experiments lacked several of these potential phosphate contacts, and the hexamers used were single-stranded under conditions of spectroscopic analysis (16). It, therefore, seems evident that the oligonucleotides employed in the spectroscopic experiments did not occupy the entirety of the endonuclease DNA binding site, thus rendering interpretation in terms of binding specificity difficult. In contrast, the results presented here are consistent with the number of phosphate contacts as deduced by alkylation methods.

REFERENCES
1. Modrich, P. (1982) CRC Crit. Rev. Biochem. 13, 287-323
2. Hedgpeth, J., Goodman, H. M., and Boyer, H. W. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 3448-3452
3. Dugaiczky, A., Hedgpeth, J., Boyer, H. W., and Goodman, H. M. (1974) Biochemistry 13, 503-512
4. Greene, P. J., Poonian, M. S., Nussbaum, A. L., Tobias, L., Garfin, D. E., Boyer, H. W., and Goodman, H. M. (1975) J. Mol. Biol. 99, 237-261
5. Modrich, P., and Zabel, D. (1976) J. Biol. Chem. 251, 5866-5874
6. Newman, A. K., Rubin, R. A., Kim, S.-H., and Modrich, P. (1981) J. Biol. Chem. 256, 2131-2139
7. Greene, P. J., Gupta, M., Boyer, H. W., Brown, W. E., and Rosenberg, J. M. (1981) J. Biol. Chem. 256, 2143-2153
8. Rubin, R. A., and Modrich, P. (1980) Methods Enzymol. 65, 96-104
9. Modrich, P. (1979) Q. Rev. Biophys. 12, 315-369
10. Lu, A.-L., Jack, W. E., and Modrich, P. (1981) J. Biol. Chem. 256, 13200-13206
11. Riggs, A. D., Bourgeois, S., and Cohn, M. (1970) J. Mol. Biol. 53, 401-417
12. Richter, P. H., and Eigen, M. (1974) Biophys. Chem. 2, 255-263
13. Berg, O. G., Winter, R. B., and von Hippel, P. H. (1981) Biochemistry 20, 6929-6948
14. Halford, S. E., and Johnson, N. P. (1980) Biochem. J. 191, 593-604
15. Woodhead, J. L., and Malcolm, A. D. B. (1980) Nucleic Acids Res. 8, 389-402
16. Goppelt, M., Pingoed, A., Mass, G., Mayer, H., Koster, H., and Frank, R. (1980) Eur. J. Biochem. 104, 101-107
17. Jack, W. E., Rubin, R. A., Newman, A., and Modrich, P. (1981) in Gene Amplification and Analysis (Chirikjian, J. G., ed) Vol. 1, pp. 155-179, Elsevier/North-Holland, New York
18. Rosenberg, J. M., Boyer, H. W., and Greene, P. J. (1981) in Gene Amplification and Analysis (Chirikjian, J. G., ed) Vol. 1, pp. 131-164, Elsevier/North-Holland, New York
19. Rubin, R. A., and Modrich, P. (1977) J. Biol. Chem. 252, 7255-7262
20. Bovi, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Hayneker, H. L., Boyer, H. W., Croo, J. H., and Falkow, S. (1977) Gene 2, 95-113
21. Hardies, S. C., and Wells, R. D. (1979) Gene 7, 1-14
22. Glynn, I. M., and Chappell, J. B. (1964) Biochem. J. 90, 147-149
23. Weiss, B., Lowry, T. R., and Richardson, C. C. (1980) J. Biol. Chem. 245, 4530-4542
24. Larson, J. E., Hardies, S. C., Patient, R. K., and Wells, R. D. (1979) J. Biol. Chem. 254, 5535-5541
25. Jack, W. E., Terry, B. J., and Modrich, P. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 4010-4014
26. Sutcliffe, J. G. (1976) Cold Spring Harbor Symp. Quant. Biol. 41, 77-90
27. Segel, I. H. (1975) Enzyme Kinetics, pp. 109-111, John Wiley and Sons, New York
28. Rudolph, B. F. (1979) Methods Enzymol. 63, 413-414
29. Todhunter, J. A. (1979) Methods Enzymol. 63, 406-407
30. Castellan, G. W. (1971) Physical Chemistry, pp. 245-247, Addison-Wesley, Reading, Massachusetts
31. Record, M. T., Jr., Lohman, T. M., and deHaseth, P. (1976) J. Mol. Biol. 107, 145-158
32. Record, M. T., Jr., deHaseth, P. L., and Lohman, T. M. (1977) Biochemistry 16, 4791-4796
33. Record, M. T., Jr., Woodbury, C. P., and Lohman, T. M. (1976) Biopolymers 15, 883-915
34. Winter, R. B., and von Hippel, P. H. (1981) Biochemistry 20, 6948-6960
35. Lohman, T. M., deHaseth, P. L., and Record, M. T., Jr. (1980) Biochemistry 19, 3522-3530
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