Measurement of Binding Constants for Protein–DNA Interactions by DNA–Cellulose Chromatography†

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ABSTRACT: We describe the application of DNA–cellulose chromatography to the determination of binding constants for the nonspecific interaction between proteins and DNA. The method involves loading a small DNA–cellulose column to a low binding density and subsequently eluting the protein with buffer at a constant salt concentration. The elution is conveniently followed by monitoring the protein fluorescence of the eluate. From the shape of the elution profile, the binding constant for the interaction can be calculated. Employing columns containing double-stranded calf thymus DNA–cellulose, we have measured binding constants in the range of 10^4 to 10^6 M^{-1}. Extension of this range is possible by varying the amount of DNA on the column. For *lac* repressor, agreement between our measurements and those of Revzin, A., and von Hippel, P. H. [(1977) *Biochemistry 16* (second of five papers in a series in this issue)], who employ an absolute boundary sedimentation method, is good. The column method should be useful for the rapid screening of the effect of a large number of variables on protein–DNA binding constants.

Knowledge of the behavior of the observed equilibrium constant K_{obsd} for a charged ligand–nucleic acid interaction as a function of ion concentrations, pH, and temperature permits one to sort out the various contributions to the free energy of complex formation (Record et al., 1976; deHaseth et al., 1977). The interactions of such ligands as Mg^{2+}, oligolysines, and RNase with nucleic acids are driven predominantly by the entropic effect of release of bound counterions (in these cases from the nucleic acid) in the association reaction (Record et al., 1976). It appears that counterion release may play a general role in the interactions of biological polyelectrolytes (Record and Manning, in preparation); this entropic effect must be considered along with the other noncovalent interactions in accounting for the stability of specific biopolyelectrolyte complexes.

To assess the contribution of the counterion release term to the reaction free energy, it is sufficient to determine the ion concentration dependence of K_{obsd} (Record et al., 1976). Unfortunately there are few simple techniques available for this purpose, particularly if the ligand itself is a macromolecule. In general, spectroscopic techniques have not proved to be sufficiently sensitive to complex formation to use as an analytical method to obtain K_{obsd}. The recent report of a fluorescent probe for the RNA polymerase–DNA interaction (Faerber and Vizethum, 1976) suggests that optical methods may prove useful in the future. Von Hippel and co-workers (Jensen and von Hippel, 1977; Revzin and von Hippel, 1977) have developed a rigorous thermodynamic method in which DNA and protein are equilibrated in a centrifuge cell, and complexes are separated from free protein by sedimentation. Since this technique requires simultaneous determination of free and bound protein concentrations, high binding densities are generally used, and the effect of overlapping binding sites on K_{obsd} (McGhee and von Hippel, 1974) must be corrected for by extrapolation to zero binding density. In the present paper, we describe the calibration and use of DNA cellulose columns to measure binding constants at low binding densities. Subsequent papers demonstrate the utility of this method in analyzing the nonspecific interactions of *lac* repressor protein and RNA polymerase with DNA (deHaseth et al., 1977; Record et al., 1977; deHaseth, Lohman, Burgess, and Record, in preparation).

The use of DNA–cellulose columns as a preparative technique for isolating and purifying DNA-binding proteins from cell lysates was introduced by Alberts et al. (1968) and Litman (1968). In their method, a partially purified lysate is applied to the column at low salt concentration and eluted with a continuous or stepwise salt gradient. In our analytical application of the method, a small DNA–cellulose column is loaded at constant salt concentration with a small quantity of pure protein, so the initial binding density is low. The protein is eluted with the same constant salt concentration. The volume required to elute a given amount of protein is related to the binding constant K_{obsd} of the protein–DNA complex. We verify the uniqueness of this relationship and, by comparison with the data of Revzin and von Hippel (1977), show that the DNA–cellulose column method provides absolute binding constants for the repressor–DNA interaction.

Materials and Methods

(a) *Reagents.* Calf thymus DNA was from Worthington; cellulose was Whatman CF 11. All other chemicals were reagent grade.

(b) *Buffers.* Buffer T is 0.01 M Tris[1] (pH 8.0 at 4 °C), 7×10^{-3} M β-mercaptoethanol, 10^{-4} M EDTA, 5% (v/v) glycerol. Buffer P is 0.01 M Na_2HPO_4 (pH 7.4 at 20 °C) instead of Tris, but otherwise the same as buffer T. In describing a buffer, the $[Na^+]$ is indicated as a subscript, e.g., $T_{0.13}$ is buffer T with a total $[Na^+]$ of 0.13 M.

(c) *lac Repressor.* Three preparations of *E. coli lac* repressor were used in this study, all purified according to Platt et al. (1973). The initial experiments were carried out on repressor from *E. coli* strain G166/BMH74-12 supplied by Drs. M.

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

Pasek and W. Gilbert (Harvard). This sample was better than 99% pure as judged by polyacrylamide gel electrophoresis, where it ran as a single band; it was fully active in operator binding, and crystallized under appropriate conditions; it behaved as a homogeneous sample in isoelectric focusing experiments (M. Pasek, personal communication). Subsequently, two samples of repressor were isolated at Wisconsin from frozen *E. coli* strain H461 cells provided by Dr. W. Reznikoff. The purity of these preparations was judged to be better than 95% from polyacrylamide gels, on which they coelectrophoresed with the Harvard sample. They were active in binding isopropyl thiogalactoside (IPTG), but were not further characterized. In most respects the interactions of the three samples of repressor with nonspecific DNA sites were the same within experimental uncertainty.

Repressor samples were stored at −20 °C at concentrations of 8–15 mg/mL in the buffer recommended by Platt et al. (1973), which contains high concentrations of Tris buffer and glycerol to prevent aggregation of the protein. Concentrations of repressor solutions were determined spectrophotometrically, using a molar extinction coefficient for the tetramer of 9×10^4 M^{-1} cm^{-1} at 280 nm (Butler et al., 1977). This value is in good agreement with that recently determined by Charlier et al. (1977). In the calculations a molecular weight of the *lac* repressor tetramer of 148 640 was used, based on the monomer molecular weight of 37 160, as deduced from the amino acid sequence (Beyreuther et al., 1975).

(d) *DNA-Cellulose Chromatography of lac Repressor.* DNA-cellulose was prepared from native calf thymus DNA and washed cellulose by the method of Alberts and Herrick (1971). Washed and defined DNA-cellulose was suspended in $T_{0.15}$ and stored frozen at −20 °C in small portions until use. No change in the properties of the DNA-cellulose was observed after storage for more than 2 years. Typically columns with a packed volume of 1.0 mL were poured in a Bio-Rad 0.7 × 4 cm polypropylene column, washed with $T_{0.7}$, and then washed extensively with $T_{0.15}$ before use. The DNA content of the washed column was determined by the procedure of Alberts and Herrick (1971) to be 150 ± 10 μg per mL of packed column volume. The characterization and calibration of these columns are described below.

Column elution experiments were performed in a cold room at 4 °C unless otherwise noted. Before the start of an experiment, the column was washed with approximately 25 mL of buffer at the salt concentration employed in the experiment. An aliquot of the repressor stock solution (typically 10 μL) containing typically 87 μg of repressor was diluted into 1 mL of the buffer used in the experiment and applied to the column. After a 1-mL rinse, the column was attached to the buffer reservoir and eluted at a constant rate (typically 17 mL/h), using a peristaltic pump attached at the bottom of the column. Approximately 25 fractions of 2.0 mL volume were collected in plastic tubes over a 3-h interval using a micro fraction collector in the drop counting mode. Subsequently the column was rinsed with 8 mL of $T_{0.7}$ to remove the protein that still remained on the column. This was again collected in 2-mL fractions. The concentration of repressor in the collected fractions was determined by measuring the intrinsic fluorescence of the repressor (excitation at 285 nm, emission at 345 nm) on an Aminco Bowman spectrofluorometer, equipped with a "ratio" photometer. Concentrations as low as 0.1 μg/mL were routinely determined. (Repressor has 2 tryptophan residues per monomeric unit, and thus has a fluorescence spectrum characteristic of that of tryptophan; cf. Laiken et al. (1972); Friedman et al. (1976).) The cuvette was thermostated at 4 °C, and polarizers were used to reduce the effect of scattered light. The fluorescence was found to be a linear function of protein concentration over the whole range of concentrations eluted. No effect of [NaCl] on the protein fluorescence was detected in the range of NaCl concentrations of 0.05–0.7 M, within the error of the measurement. We have found that dilute repressor solutions are very sensitive to loss of material (as judged by fluorescence) upon agitation of the solution. The reading on a 5 μg per mL solution dropped to less than 10% of the original value upon mixing for 2 min at medium speed on a vortex mixer. An unagitated solution showed no change in reading over the same period. A study with solutions of different concentrations of *lac* repressor showed that the effect was relatively more important for dilute solutions; prerinsing the plastic tubes with the repressor solution diminished the effect. In view of this phenomenon, care was taken to handle all fractions in a standardized way, as some agitation during collection of the fractions and transfer to the cuvette was unavoidable.

Elution data are presented as the fraction (or, alternatively, percentage) of the total amount of applied protein which eluted in a particular 2-mL fraction. This was obtained from the quantity $F_i/\Sigma F_i$, where F_i is the fluorescence of fraction i and ΣF_i is the total fluorescence of all fractions, including the $T_{0.7}$ wash. Within an experimental uncertainty of ±15%, the quantity ΣF_i has the value expected for the amount of repressor applied to the column.

Each column was used several times; no DNA loss could be detected from columns which had been used up to seven times (over the course of 7 days), in agreement with Alberts and Herrick (1971) who report that the disentanglement of DNA from the cellulose is very slow at low temperature.

(e) *Determination of the Binding Capacity of a DNA-Cellulose Column for lac Repressor.* Previous application of DNA-cellulose chromatography had shown that column-bound DNA had a smaller capacity for binding RNA polymerase than did DNA which was free in solution (Bautz and Dunn, 1971; deHaseth, Lohman, Burgess, and Record, in preparation). It was therefore necessary to determine the binding capacity of DNA-cellulose for *lac* repressor. A 0.5-mL column containing 75 μg of DNA was equilibrated in low salt $T_{0.05}$ buffer with excess repressor (1.75 mg of repressor), and washed with 40 mL of the same buffer to remove unbound repressor. We estimate that $K^{RD}_{obsd} \simeq 10^9$ M^{-1} in $T_{0.05}$ by extrapolation of the data of Figure 7 (see below). Therefore little bound repressor was eluted by the washing procedure. In a subsequent high salt ($T_{0.7}$) wash to remove DNA-bound repressor, 0.70 mg of repressor was eluted. Assuming that each repressor molecule renders approximately 22–24 nucleotides inaccessible to other repressor molecules (Maurizot et al., 1974; Butler et al., 1977), we estimate that 46% of the total amount of DNA on the column is able to participate in the binding reaction. For a 1-mL column (the size routinely used), this amounts to 2.1×10^{-7} mol of DNA nucleotides. The reproducibility of this result was ±10%. A small correction should be applied to account for the difficulty in achieving complete saturation of the DNA lattice by the large repressor ligand (cf. McGhee and von Hippel, 1974); this amounts to a 3% increase in the amount of available DNA and was neglected in comparison with the experimental uncertainty.

Results and Discussion

(a) *Elution of lac Repressor from a DNA-Cellulose Column.* Figures 1 and 2 illustrate the elution of *lac* repressor from a 1-mL DNA-cellulose column initially loaded with 87 μg of repressor. If bound uniformly throughout the column, this would correspond to a binding density ν of 2.8×10^{-3} mol of

repressor bound/mol of accessible DNA nucleotides, or a maximum coverage of less than 7% of the available DNA. (Recall that 1.4 mg of repressor would be required for saturation of this 1-mL column.) We define the observed association constant for the nonspecific interaction of *lac* repressor with DNA as

$$K^{RD}_{obsd} = \frac{[RD]}{[R][D]} \quad (1)$$

where [RD] is the concentration of the repressor–DNA complex, and [R] and [D] are the concentrations of free repressor and free DNA nucleotides, respectively. Because so little of the DNA is covered with protein under our experimental conditions, we not only can neglect effects of site overlap (McGhee and von Hippel, 1974) but also can approximate [D] by the total accessible nucleotide concentration. Then rearrangement of eq 1 yields

$$\nu = K^{RD}_{obsd}[R] \quad (2)$$

where ν is the ratio of the concentration of the RD complex to the total nucleotide concentration. Equation 2 is equivalent to the linear isotherm characteristically assumed in the theory of chromatography (Martin and Synge, 1941).

Figures 1 and 2 show the elution characteristics of these small DNA–cellulose columns. In Figure 1, the percentage of total protein eluting in a given 2.0-mL fraction is plotted against the number of that fraction for columns eluted at 0.12, 0.14, and 0.16 M NaCl. There is an initial transient period, corresponding to the first one or two fractions to contain protein, in which 5–10% of the applied protein elutes from the column. This effect is visible in the curve at 0.12 M Na^+ in Figure 1; it may result from the loading procedure used, or may reflect the presence of a component in the sample that is unable to bind to DNA (possibly *lac* repressor aggregates). Subsequent to this initial period, which also corresponds to the retardation period of repressor on a cellulose column without DNA or on a DNA–cellulose column eluted at very high salt (0.7 M NaCl, data not shown), repressor elutes gradually from the column. This behavior is quite unlike that of a conventional chromatogram.

A more useful representation of the elution is given in Figure 2, where the percentage of total protein remaining on the column after elution of a fraction is plotted as a function of the index of that fraction. A family of approximately exponential decay curves is obtained from the data of Figure 1 and additional experiments at other NaCl concentrations. From Figure 2 it is clear that the elution profile must be related to the binding constant K^{RD}_{obsd}. K^{RD}_{obsd} is known to increase dramatically with decreasing salt concentration, as is the case generally for the interactions of positively charged ligands with nucleic acids (Record et al., 1976; Revzin and von Hippel, 1977). As the salt concentration is reduced in Figure 2, the duration of the elution increases, and the slope of the elution profile at any fixed volume of elution decreases. The elution profile is independent of flow rate over the range investigated (11–27 mL/h) and is reproducible under controlled conditions of salt concentration, pH, and temperature (deHaseth et al., 1977).

(b) *The Relationship of the Elution Profile to K^{RD}_{obsd}.* Our motivation in these studies was the development of a simple and rapid technique to measure relative and, if possible, absolute binding constants for interactions of charged ligands with DNA. Figure 2 indicates that a relationship must exist between the elution curve and the binding constant. Here we propose a simple form for that relationship, which gives results in agreement with those obtained by the thermodynamically

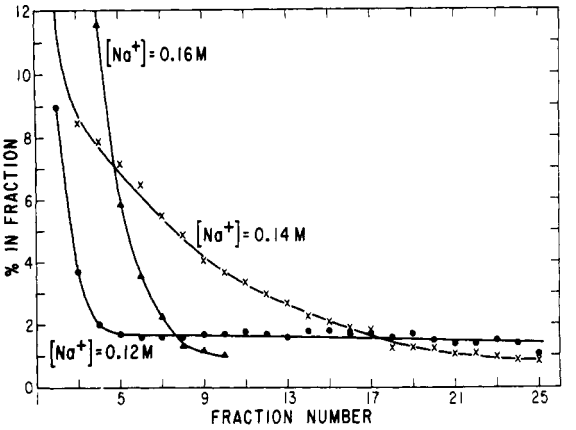

FIGURE 1: The elution of *lac* repressor from DNA–cellulose columns. Repressor (87 μg; 100 μg for the elution at 0.16 M NaCl) was loaded and eluted with buffer T. NaCl concentrations of the elution buffers are indicated on the graph.

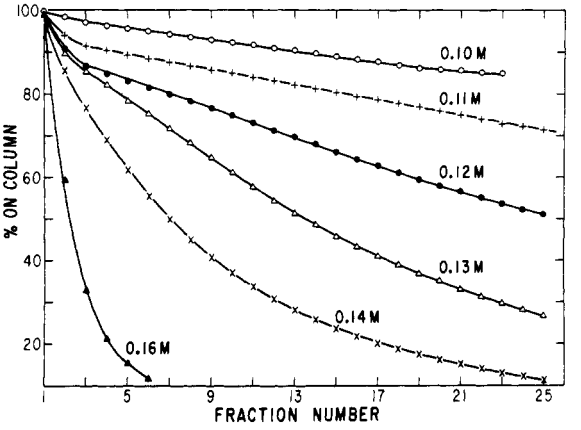

FIGURE 2: Retention of *lac* repressor on DNA–cellulose columns as a function of fraction number; linear plots. Columns were loaded with 87 μg of *lac* repressor (100 μg for the elution at 0.16 M NaCl) and eluted with buffer T. NaCl concentrations of the elution buffers are indicated on the graph.

rigorous technique of Revzin and von Hippel (1977).

We define $P_{c,i}$ as the percentage of total protein remaining on the column after elution of fraction i, and define $P_{f,i}$ as the percentage of total protein eluted in fraction i. Figure 3 shows the data of Figure 2 plotted as log $P_{c,i}$ vs. i. We observe a linear dependence over the range of applicability of the column technique; the elution profiles in Figure 2 are accurately described by single exponential decays. Hence each profile can be characterized by a single parameter (or decay constant).

Moreover, we find that $P_{f,i}$ is a linear function of $P_{c,i}$, as shown in Figure 4. In fact, $P_{f,i}$ is seen from Figure 4 to be very nearly proportional to $P_{c,i}$; the assumption that 5–10% of the protein on the column is bound much more strongly than the rest and can be neglected in the analysis would give an exact proportionality between $P_{f,i}$ and $P_{c,i}$.

Let us assume

$$P_{f,i} = kP_{c,i} \quad (3)$$

where k is a proportionality constant. This assumption allows us to explain the linearity of semilogarithmic plots of $P_{c,i}$ vs. i (cf. Figure 3), which provide a more convenient measure of the quantity k. In addition, the proportionality of $P_{f,i}$ to $P_{c,i}$ is not unlike the behavior expected for a linear binding isotherm. For certain models of the elution process k can be related to the binding constant for the protein–DNA interaction.

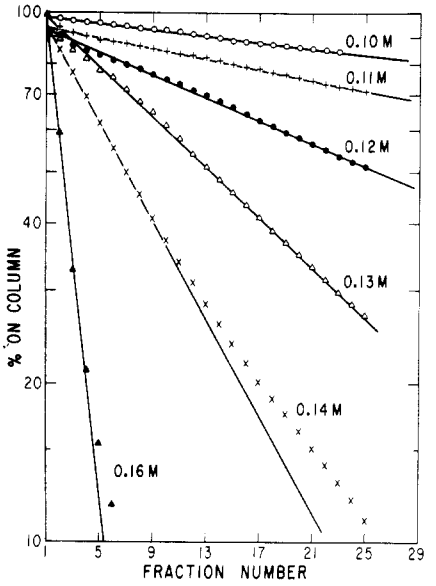

FIGURE 3: Retention of *lac* repressor on DNA-cellulose columns as a function of fraction number; semilog plots. Data from Figure 2. NaCl concentrations of the elution buffers are indicated on the graph.

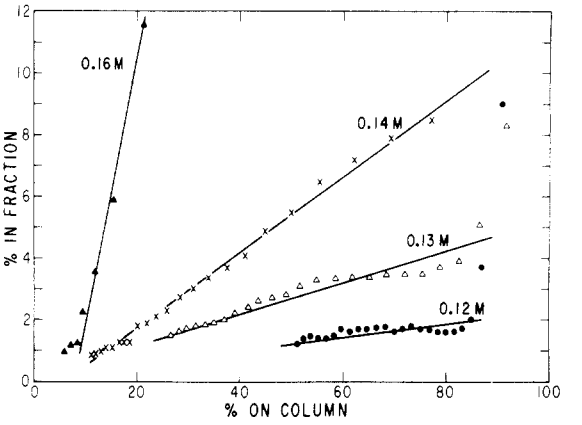

FIGURE 4: Proportionality between the amount of *lac* repressor eluted and the amount remaining on the column. Selected data from Figure 2. NaCl concentrations of the elution buffers are indicated on the graph.

(See below.) Comparison of the binding constants so obtained with the results of Revzin and von Hippel (1977) for *lac* repressor, as well as preliminary measurements on Mg^{2+} (Erdmann, deHaseth, and Record, work in progress), indicate the applicability of this description.

By conservation of ligand, we obtain

$$P_{c,i} = P_{c,0} - \sum_{j=1}^{i} P_{f,j} \tag{4}$$

where j is an index of fractions. By repeated application of eq 3 to eq 4 and inserting $P_{c,0} = 100\%$, we find

$$\frac{P_{c,i}}{100} = \left(\frac{1}{1+k}\right)^{i} \tag{5}$$

or

$$\log \frac{P_{c,i}}{100} = -i \log (1+k) \tag{6}$$

Consequently plots of $\log P_{c,i}$ vs. i should be linear with slope $-\log (1+k)$, from which k is obtained.

We do not have a rigorous formulation of the relationship between the parameter k which describes the elution profile and the binding constant K^{RD}_{obsd} of the protein-DNA interaction. Two limiting situations can be considered, however, in which such a relationship can be derived. Although we cannot prove the general applicability of either case to the conditions of our experiments, they provide a means of estimating K^{RD}_{obsd} from k. These cases are (a) the discrete extraction process, and (b) the uniform or one theoretical plate column. Here we will consider case a in some detail.

If the elution of the column, a continuous extraction process, were approximated by a discrete extraction of the column matrix with each fraction, then $P_{c,i}$ is proportional to ν_i, the average density of protein in equilibrium with free ligand at concentration R_i in the extraction step. Letting D_T designate the total molar amount of accessible DNA nucleotides on the column, w the weight of protein of molecular weight M initially applied to the column, and V_f the volume of the fraction, we have

$$R_i = \frac{P_{f,i}}{100} \frac{w}{M} \frac{1}{V_f} \tag{7}$$

and

$$\nu_i = \frac{P_{c,i}}{100} \frac{w}{m} \frac{1}{D_T} \tag{8}$$

Then, when ν_i is sufficiently small to use the linear isotherm approximation (eq 2) in conjunction with eq 3, we find that

$$K^{RD}_{obsd} = \frac{\nu_i}{R_i} = \frac{V_f}{kD_T} \tag{9}$$

For our standard 1-mL column, $D_T = 2.1 \times 10^{-7}$ mol (see above). The standard fraction volume was 2.0 mL. Therefore $K^{RD}_{obsd} = 9.5 \times 10^3/k$ in units of M^{-1}.

Equations 6 and 9 can also be derived if the column is considered to perform no fractionation, being at equilibrium everywhere so the distribution of bound protein is uniform during the elution; this is the one theoretical plate limit of the theory of chromatography of Martin and Synge (1941) (Anderson and Record, to be published). Even if the column consists of multiple theoretical plates, as is more likely to be the case, it can be shown that eq 6 and 9 are valid in the region of the elution where the column is uniform (Anderson and Record, to be published). Since the column is loaded under the reversible conditions of the elution experiment, we anticipated that the protein would be distributed throughout the column early in the elution. Furthermore, the density of bound protein in an initially nonuniform column should be greater toward the top of the column. Elution of the column would then tend to create a more uniform distribution during the run. An experiment was performed to test this supposition. An elution in $T_{0.13}$ (0.13 M NaCl; cf. Figure 2) was interrupted after five fractions when 40% of the total protein had eluted from the column. The upper and lower regions of the column were separated and individually extracted with aliquots of $T_{0.7}$ buffer. Protein concentrations in these regions (at the 60% point in the elution) were determined by fluorescence. We found 30% of the total protein in the top 45% of the column, and 30% in the lower 55% of the column. Consequently in mid-elution in the mid-range of the column technique, the column is close to uniform, and the above analysis should be applicable.

We have not checked the uniformity of the column under other ionic conditions, or at other points in the elution profile. Although we do not believe that either the discrete extraction process or the uniform column is a rigorous model of the elution process, we have shown that, under the conditions of our experiments, a single parameter, k, characterizes the exponential elution profile, and that k can plausibly be related to K^{RD}_{obsd},

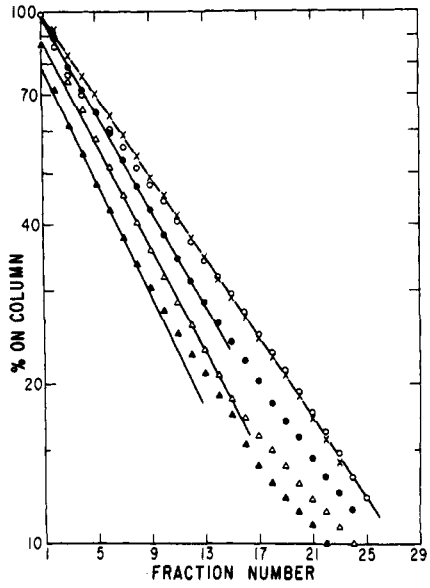

FIGURE 5: The dependence of the elution of *lac* repressor from DNA cellulose columns on the amount of protein loaded; semilog plots. Amounts loaded were: 43 μg (○), 87 μg (X), 87 μg (●), 130 μg (△), and 174 μg (▲). Elution with $T_{0.13}$.

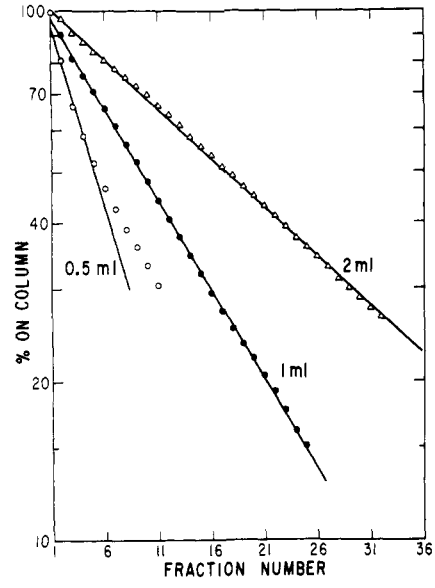

FIGURE 6: The dependence of the elution of *lac* repressor from DNA-cellulose on the volume (or total DNA content) of the columns; semilog plots. The column volumes are indicated on the graph. The columns were loaded to the same initial binding density with the following amounts of protein: 0.5-mL column, 43 μg; 1-mL column, 87 μg; 2-mL column, 174 μg. Elution with $T_{0.13}$.

the association constant. In a subsequent section we will show that binding constants K^{RD}_{obsd} calculated by the column technique for *lac* repressor are in reasonable agreement with published values, thereby providing a calibration of the method.

As mentioned above, small deviations from an exponential decay of the amount of protein remaining on the column are frequently observed in the initial stages of the elution. In some cases (cf. the data at 0.14 M Na^+), deviations from exponential behavior also occur late in the elution profile, where 30–40% or less of the original protein still remains on the column. This effect, which does not interfere significantly with the determination of k from the experiment, may result from systematic cumulative errors in the fluorescence measurements, or may relate to the agitation-induced loss of repressor fluorescence described above. In experiments with RNA polymerase (deHaseth, Lohman, Burgess, and Record, in preparation), continuous curvature is always noted in plots of log $P_{c,i}$ vs. i, and the elution profiles cannot be fit by a single exponential decay. (With repressor, the tendency toward such curvature varied from preparation to preparation, although it was never a major effect. The preparation originating from Harvard gave somewhat more curvature than those subsequently prepared here.) We interpret large deviations from exponential behavior as an indication of heterogeneity in K_{obsd}, and not as an artifact of the column technique, based on the control experiments shown in this paper and on our unpublished experiments with the model ligand, Mg^{2+}, which also elutes exponentially (Erdmann, deHaseth, and Record, work in progress).

(c) *The Dependence of k and K^{RD}_{obsd} on the Column Parameters D_T and ν_0.* Equation 9 predicts that k, the parameter characterizing the column elution profile, should be inversely proportional to D_T, the total amount of accessible DNA sites, and should be independent of ν_0, the initial binding density. Then K_{obsd} will be independent of D_T and ν_0, as an equilibrium constant must be. (Note of course that the column will presumably perform chromatography at a large enough value of D_T, and that the linear isotherm would not be a valid approximation in calculating K^{RD}_{obsd} at high ν_0. Under such conditions eq 9 would not be expected to apply.)

Figures 5 and 6 illustrate the dependences of k on ν_0 and D_T in a range encompassing the usual values of these parameters in our experiments. In Figure 5, a series of elutions of *lac* repressor was performed in $T_{0.13}$ at different initial binding densities, ν_0. In this buffer, $K^{RD}_{obsd} \simeq 10^5\ M^{-1}$ (see below). Therefore more than 95% of the protein applied to the column should initially bind (at densities in the range of applicability of the linear isotherm), and ν_0 can be estimated from the total amount of protein applied. Values of ν_0 were in the range $1.4\text{–}5.6 \times 10^{-3}$, equivalent to coverage of 3–13% of the DNA sites. The corresponding values of k range from 0.09 to 0.13, respectively; from eq 9, the corresponding values of K^{RD}_{obsd} range from 1×10^5 to $7 \times 10^4\ M^{-1}$, respectively. Two slight trends are apparent with increasing ν_0: the percentage of protein not retained at all by the column increases slightly (thereby causing the vertical displacement of the lines of Figure 5), and the values of K^{RD}_{obsd} decrease slightly. A total of seven experiments carried out at $\nu_0 = 2.8 \times 10^{-3}$ (two of which are shown in Figure 5) gave $K^{RD}_{obsd} = 1.3 \pm 0.3 \times 10^5\ M^{-1}$. We conclude that there may be a slight dependence of the calculated binding constant on ν_0, but that no corrections are warranted within the limits of reproducibility of the technique.

Figure 6 shows the dependence of the elution profile on the total amount of DNA on the column. Columns with volumes of 0.5, 1.0, and 2.0 mL were loaded to the same initial binding density and eluted with $T_{0.13}$. From Figure 6, the values of k for the three columns are 0.168, 0.082, and 0.043, respectively; the corresponding values of K^{RD}_{obsd} are 1.1×10^5, 1.2×10^5, and $1.1 \times 10^5\ M^{-1}$. We consider this a strong indication that the column is not performing chromatography, and that our method of analysis is basically correct.

(d) *Calibration of the Chromatographic Method.* Figure 7 compares binding constants K^{RD}_{obsd} determined by the column method at 20 °C in phosphate buffer (pH 7.37 ± 0.03) at various NaCl concentrations with the data of Revzin and von Hippel (1977) obtained by a sedimentation method (which is thermodynamically rigorous) in phosphate buffer, pH 7.5, 20 °C. After their values of log K^{RD}_{obsd} are corrected by +0.26 log unit to account for the pH difference in the two experiments

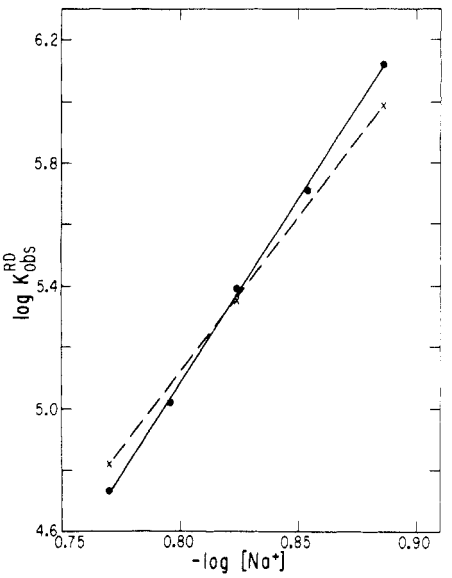

FIGURE 7: Comparison of *lac* repressor DNA binding constants obtained using analytical DNA-cellulose chromatography (O), with published values obtained using a boundary velocity sedimentation technique (X) (Revzin and von Hippel, 1977). Elution at 20 °C. See text.

(deHaseth et al., 1977) and by −0.3 log unit to express them in units of nucleotides, rather than nucleotide pairs, agreement between the two sets of data is excellent. Individual values of log K^{RD}_{obsd} agree to within ±0.15 log unit, which is within the usual limits of reproducibility of the column technique (deHaseth et al., 1977).[2] Both sets of data give linear log–log plots. The slopes of the least-squares lines illustrated in Figure 7 differ by approximately 20%, being −10.0 for the data of Revzin and von Hippel (1977) and −12.0 for our experiments.

(e) *The "Window" of the Method.* The useful range (or "window") of the column method is best discussed with reference to Figure 2. At high binding constants (low salt) the applicability of the method becomes limited by the technical problem of measuring the fluorescence of the small amount of protein eluted in each fraction. At low binding constants (high salt) the resolution is lost because the elution profiles approach those observed when a column without DNA is used. We consider the curves at 0.1 M NaCl ($K^{RD}_{obsd} \simeq 1.5 \times 10^6$ M^{-1}) and 0.16 M NaCl ($K^{RD}_{obsd} \simeq 1.4 \times 10^4$ M^{-1}) to bracket the useful range of the method, which thus covers a range of about two orders of magnitude in K^{RD}_{obsd}. The range can be extended toward lower binding constants by employing columns containing a larger concentration of DNA than those used in the present study as can be seen from eq 9. Extension in the opposite direction is more limited because of the high initial binding densities that the use of a column containing less DNA would necessitate.

Conclusions

We conclude that the elution of *lac* repressor protein, present initially at low density on a DNA-cellulose column, is exponential, and that the characteristic elution parameter k is directly proportional to the binding constant K^{RD}_{obsd} under these elution conditions. In preliminary experiments, we observed similar behavior in the elution of Mg^{2+} as ligand with NaCl solutions (Erdmann, deHaseth, and Record, work in progress). Moreover the binding constants for the Mg^{2+}–DNA interaction so obtained, and their dependence on the $[Na^+]$, appear to be in reasonable agreement with the results of other investigations (Krakauer, 1971, Clement et al., 1973).

We believe that DNA-cellulose chromatography, as employed here, is a useful addition to the available techniques for determining protein–DNA binding constants. The method is relatively fast and does not require large amounts of protein. It should prove particularly applicable in screening the effect of a large number of variables on the observed binding constant for the interaction of a protein with DNA. It is to this end that we have employed the technique to probe the nonspecific interaction of *lac* repressor and RNA polymerase with DNA. These studies are the subject of subsequent papers (deHaseth et al., 1977; Record et al., 1977; deHaseth, Lohman, Burgess, and Record, in preparation).

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[2] In view of the extreme sensitivity of K^{RD}_{obsd} to variations in salt concentration and pH, the agreement between our results and those of Revzin and von Hippel (1977) is perhaps fortuitously good.

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Nonspecific Interaction of *lac* Repressor with DNA: An Association Reaction Driven by Counterion Release†

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ABSTRACT: We have investigated the nonspecific interaction of *lac* repressor protein with DNA by a quantitative application of DNA-cellulose chromatography (deHaseth, P. L., et al. (1977), *Biochemistry 16* (third of five papers in a series in this issue)). The observed association constant for the interaction, K^{RD}_{obsd}, is a sensitive function of ion concentrations and pH. Application of binding theory to interpret these effects gives the results that 11 ± 2 monovalent ions are released in the interaction and two groups on repressor must be protonated for repressor to bind to DNA. We argue that much of the ion release results from the displacement of cations from the DNA, and estimate on this basis that 12 ± 2 phosphates are involved in ionic interactions with the protein. Ion release drives the protonation reaction and the overall repressor-DNA interaction. The major role of low molecular weight ions in the repressor-DNA interaction suggests that ion concentration changes must be considered in discussing mechanisms of control of gene expression.

Many of the association reactions of proteins are driven by the release of structured water and show the thermodynamic behavior ascribed to the hydrophobic effect (Lauffer, 1974; Tanford, 1973). A different principle is operative in the interactions of highly charged biopolymers. Analysis of the interaction of various charged ligands (Mg^{2+}, oligolysines, RNase) with nucleic acids (Record et al., 1976a) demonstrated that these association reactions are driven by the entropic effect of release of monovalent cations from the nucleic acid. The entropic effect of ion release plays a major role in the aggregation of myosin (Josephs and Harrington, 1968) and of *Escherichia coli* RNA polymerase (Wensley and Record, in preparation), and is a factor in the allosteric transition of hemoglobin (Shulman et al., 1975) and the helix coil transitions of nucleic acids (Record et al., 1976b).

Here we give an experimental study and theoretical analysis of the thermodynamics of the nonspecific interaction of *lac* repressor protein with DNA.[1] The analysis is general and should be applicable to any charged ligand-nucleic acid interaction. We find that the fundamental driving force for the association reaction is the release of a large number of counterions from the nucleic acid and perhaps also from the protein. Counterion release, we will show, drives a protonation reaction of repressor which is necessary for formation of the nonspecific complex. The implications of this study for the repressor-operator interaction are considered in the following paper (Record et al., 1977).

The participation of a large number of ions in the repressor-DNA interaction means that the extent of binding of repressor to nonspecific DNA sites is extremely sensitive to small changes in ionic conditions. It seems plausible to suggest that ion concentration changes in vivo may play some role in modulating the control of gene expression.

Theoretical Section

Consider the interaction of repressor with nonoperator DNA to form the nonspecific complex RD. The observed reaction is

$$R + D \overset{K^{RD}_{obsd}}{\rightleftharpoons} RD \qquad (1)$$

where

$$K^{RD}_{obsd} = \frac{[RD]}{[R][D]} \qquad (2)$$

and where [R] and [D] are *total* molar concentrations of free repressor and free DNA nucleotides, irrespective of titration state or degree of ligation of low molecular weight ions.

The association constant K^{RD}_{obsd} is found to be a function of temperature, pH, and monovalent and divalent ion concentrations in the reaction mixture (see below; also Revzin and von Hippel, 1977). These dependences indicate that small ions and titrated forms of R and/or D participate in the molecular association reaction. Analysis of the ion and pH dependences of K^{RD}_{obsd} using binding theory (Wyman, 1964; Schellman, 1975) allows one to obtain the numbers of participating ions, and provides information about the molecular details of the binding reaction. For example, Riggs (1971) has analyzed the pH dependence of the binding of 2,3-diphosphoglycerate to hemoglobin to determine the pK and number of titrating groups in this reaction and has shown that the apparent enthalpy of the observed association reaction results primarily from the large enthalpies of titration of these groups. An analogous approach is common in steady-state enzyme kinetics, where the pH dependence of the reaction velocity pro-

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[1] Preliminary accounts of this work were presented to the Biophysical Society (Seattle, 1976; New Orleans, 1977) and at the Cold Spring Harbor meeting on Molecular Aspects of *lac* Operon Control (1976). Some of our present conclusions supercede the results of those preliminary theoretical analyses.