Direct Measurement of Association Constants for the Binding of *Escherichia coli lac* Repressor to Non-Operator DNA†

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ABSTRACT: Binding parameters for the interaction of *lac* repressor with non-operator DNA have been determined using a sedimentation velocity technique. Analytical ultracentrifugation is used to separate DNA–protein complexes from unbound protein, thereby permitting direct optical determination of the concentration of free protein as a function of input DNA and repressor concentrations. The method yields absolute values for the association constant (K) for proteins binding nonspecifically to nucleic acid lattices; the binding site size (n), and the binding cooperativity parameter (ω) can also be estimated by this approach. Values of K for the binding of repressor to non-operator DNA have been determined under a variety of solvent conditions. At 0.15 M Na^+, 20 °C, pH 7.5, the average value of K for repressor binding to native phage λ DNA is 2.4×10^5 M^{-1} (DNA concentration in base pairs, protein in repressor tetramers). Binding is very ionic strength dependent; we find that $\delta \log K/\delta \log [Na^+] \simeq -10$. Analysis of the ionic strength dependence by the method of Record, M. T., Jr., Lohman, T. M., and de Haseth, P. ((1976), *J. Mol. Biol. 107,* 145) indicates the formation of ~11 ion-pair bonds between DNA phosphates and basic amino acid residues per repressor tetramer bound. Repressor binding is moderately pH dependent, the value of K decreasing as the pH increases from 7.2 to 7.8. Thus one or more histidine residues may be involved in the binding. The binding affinity also decreases with increasing temperature, and is somewhat increased in 30% glycerol. Under comparable ionic conditions binding to poly[d(A-T)] is ca. sixfold tighter than to phage λ (or calf thymus) DNA. It is shown directly that the binding of inducer (IPTG) to repressor does not change the repressor–DNA association constant. However "core" repressor, produced by brief digestion of the protein with trypsin to remove the N-terminal 58 residue peptide from each subunit, does not bind to DNA. The repressor–DNA binding data are consistent with a site size (n) of about 12 base pairs covered per repressor molecule bound (assuming binding to only one side of the double-helical DNA lattice), and show that binding involves no protein–protein cooperativity ($\omega = 1$).

In the preceding paper (Butler et al., 1977), we presented and discussed measurements of the site size for the binding of *lac* repressor to non-operator DNA. In addition, some aspects of the repressor–inducer interaction were considered. In this paper we report the results of direct determinations of the binding constants of *lac* repressor to various non-operator DNAs and DNA models as a function of environmental variables (including ionic strength, pH, temperature, etc.). These measurements provide some of the physicochemical information needed to permit quantitative interpretation of the role of non-operator DNA binding of repressor and inducer-complexed repressor in controlling the function of the lactose operon in vivo (von Hippel et al., 1974; Kao-Huang et al., 1977).

Materials and Methods

Materials. Most of the materials used in this study have been described in the accompanying paper (Butler et al., 1977). Poly[d(A-T)] was purchased from Miles and deproteinized by phenol extraction. The sedimentation coefficient ($s_{20,w}$) of this polynucleotide was 11 S, and concentrations were determined using an extinction coefficient (ϵ_{262}) of 13.3×10^3 M^{-1} cm^{-1} (per mole base pairs; Inman and Baldwin, 1962).

Repressor was isolated and purified as described in Butler et al. (1977); repressor concentrations were determined using an extinction coefficient (ϵ_{280}) of 2.25×10^4 M^{-1} cm^{-1} *per repressor subunit.* Extinction coefficients for repressor at other wavelengths were determined from this value via spectra measured in a Cary 15 spectrophotometer on monodisperse (nonaggregated) solutions. All experiments reported in detail here were performed with a single preparation of repressor from an overproducing (i^{SQ}) strain; however, similar data have been obtained with several other i^{SQ} and i^Q preparations (see Butler et al., 1977).

Phage λ DNA was isolated from the *E. coli* (K12) strain RV80, containing two temperature inducible λ lysogens. One prophage ($\lambda C_{1857}S_{t68}h80dlac^+$) carries the *lac* genes, but is unable to produce complete phage. The "helper" prophage ($\lambda C_{1857}S_{t68}h80$) permits isolation of a mixture of whole viral particles, one type carrying the *lac* genes, the other not. These phage have different buoyant densities and are separated on a CsCl gradient. The non-*lac*-containing phage λ DNA was used in these studies. A possible slight contamination with lac^+ DNA represents no problem in direct measurements of nonspecific binding, since each λ DNA molecule has many thousands of potential repressor binding sites and thus the presence or absence of the operator region is not observable. The phage λ DNA preparations had $s_{20,w}$ values of 22–24 S, and thus consisted almost entirely of native whole and half λ DNA molecules. Therefore perturbation of binding isotherms due to possible end effects should be negligible and has been ignored in the analysis. Concentrations of phage λ DNA were determined using an extinction coefficient (ϵ_{260}) of 13.3×10^3 M^{-1} cm^{-1} (per mole base pairs).

Repressor–DNA Binding Parameters by Analytical Ultracentrifugation. Association constants (K) and binding site

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sizes (n) were determined using a modification of a preparative ultracentrifuge boundary sedimentation technique described elsewhere (Jensen and von Hippel, 1977). The technique yields absolute binding constants, and no assumptions about the nature of the interaction are required. Some preliminary measurements of *lac* repressor–DNA binding (von Hippel et al., 1975) were performed using the preparative ultracentrifugation technique; here we have adapted this procedure to a Beckman Model E analytical ultracentrifuge equipped with photoelectric scanner and UV[1]-absorption optics.

Preparation of Solutions. Repressor tends to aggregate on standing. This aggregation apparently does not involve disulfide bond formation, since it occurs equally in the presence or absence of dithiothreitol. Aggregation can be rapid at low ionic strength (<0.1 M Na^+), but does not occur if the repressor is bound to DNA. Since a typical set of binding experiments required to establish a binding isotherm involves six or more centrifugation runs over the course of a day, the following procedure was used routinely to minimize aggregation-based difficulties in the measurement.

Freshly thawed stock repressor was mixed with DNA to produce a solution with the desired DNA/repressor ratio at approximately the desired component concentrations. This solution was then dialyzed overnight into buffer containing 0.01 M Na_2HPO_4, 0.0001 M Na_2EDTA; under these ionic conditions all repressor is bound to the DNA (see below). On the following day the repressor–DNA solutions were held at 0.02 M Na^+ until just before a run, and then brought to the desired ionic strength by addition of 2 M NaCl. The solution was placed in the solute sector of a Model E centrifuge cell, and the reference sector filled with buffer. The cell was then put into a Cary 15 spectrophotometer to record (against an air "blank") the absorbance spectrum of the DNA–protein solution (over the range $230 < \lambda < 340$ nm), and also the spectrum of the buffer solution; the difference spectrum is that of the DNA–protein sample. (Appropriate corrections, if necessary, were made for optical density due to light scattering, using the absorbance at 340 nm.)

The DNA–protein spectrum was then dissected via two wavelength analysis to obtain the true input concentrations of DNA (C^{DNA}) and of repressor (C^R) in each solution. [Extinction coefficients at 265 nm (ϵ_{265}^R, ϵ_{265}^{DNA}) and at 235 nm (ϵ_{235}^R, ϵ_{235}^{DNA}) had been determined as described above.] Input concentrations were evaluated by solving the simultaneous equations:

$$A_{235} = \epsilon_{235}^R C^R + \epsilon_{235}^{DNA} C^{DNA}$$

$$A_{265} = \epsilon_{265}^R C^R + \epsilon_{265}^{DNA} C^{DNA}$$

The concentrations so determined were in good agreement with those expected from the known inputs of DNA and repressor.

This procedure permits accurate comparisons of K values under different solvent conditions. For example, to compare binding at 0.13, 0.15, and 0.17 M Na^+, enough DNA–repressor solution at a particular DNA/R ratio was made for three runs; one aliquot was adjusted to 0.13 M Na^+ and run in the centrifuge, and then, in turn, the remaining aliquots were made 0.15 and 0.17 M Na^+ and centrifuged. In this way two points on each of three Scatchard (1949) plots could be generated in a single day, and regardless of experimental errors (in particular those involved in pipetting of protein solutions), each trio of corresponding points on the Scatchard plots was derived from solutions having exactly the same DNA/R ratio. Similar procedures were followed wherever possible to facilitate comparison of data between runs involving variation of other parameters.[2]

Calibration of the Model E Optical System. The ultraviolet absorbance system of the Model E ultracentrifuge utilizes a Beckman DU monochromator, and requires that the slits be wide open to provide adequate illumination. Therefore, because of stray light errors, absorbance readings in the Model E had to be calibrated against values measured in the Cary 15 spectrophotometer. To this end the spectrum of a DNA solution in a centrifuge cell was determined in the Cary 15 as described above. This solution was then centrifuged at a relatively low speed (e.g., 16 000 rpm), and absorbance values at 280, 265, and 235 nm were recorded by rapid scanning at each wavelength before the DNA had moved appreciably from the meniscus. This procedure was repeated with a solution of repressor. The calibration ratios (absorbance value in Cary 15 divided by absorbance value in Model E) were the same for DNA and for repressor at 280 nm and at 265 nm. At 235 nm the ratios were 1.356 with DNA in the centrifuge cell, and 1.673 with repressor; this difference results because the large slit opening in the Model E optical system has more pronounced effects when the absorbance of the sample changes markedly over a narrow wavelength range (e.g., for proteins at $\lambda < 245$ nm). Mixtures of protein and DNA yielded appropriate intermediate calibration ratios at 235 nm.

Sedimentation Experiments. An 0.4 to 0.5 mL aliquot of DNA–protein solution at ionic strength 0.02 M (Na^+) was brought to the desired ionic conditions by addition of concentrated NaCl, $MgCl_2$, etc., and immediately transferred to a standard double sector Model E analytical ultracentrifuge cell. Following measurement of the absorbance of the solution in the Cary 15, the cell was inserted into an An-D rotor, the rotor brought to 24 000 rpm, and the cell rapidly scanned at 265, 280, and 235 nm. These initial scans, taken before the DNA–protein complexes had moved very far down the cell, provided a check on the state of the solution. For example, in some runs containing added Mg^{2+} such scans revealed that all of the DNA and protein had aggregated and precipitated out of solution.

Figure 1 illustrates results from a typical experiment. After scanning at a slower rate at 265 and 235 nm for about 15 min to follow the sedimentation of the DNA–protein complexes, the rotor was brought to 40 000 rpm and centrifuged for an additional 60 min, i.e., until the unbound repressor had moved completely away from the meniscus. This allowed establishment of a good base line for determination of A_{235} for the free protein plateau, from which the concentration of free protein was evaluated. We note that the protein plateau is virtually flat, indicating that no appreciable redistribution of free protein occurs as a consequence of the sedimentation of the DNA.

Filter Binding Measurements. The filter assay method of Riggs et al. (1968) was used to monitor the operator-binding activity of each repressor preparation. Schleicher and Schuell B-6 nitrocellulose filters, binding buffers, and other materials and procedures were utilized as described by Riggs et al.

[1] Abbreviations used: UV, ultraviolet; Tris, tris(hydroxymethyl)aminomethane; Na_2EDTA, disodium ethylenediaminetetraacetate.

[2] The addition of NaCl to the 0.01 M Na_2HPO_4, 0.001 M Na_2EDTA buffer causes a change in solution pH. If the 0.01 M Na_2HPO_4 solution is at pH 7.78, the pH shifts to 7.52 when NaCl is added to give 0.13 M Na^+ total; it shifts to 7.50 at 0.15 M Na^+ total and to 7.48 at 0.17 M Na^+ total. For present purposes we consider each of these three solutions to be at pH 7.5—the variation in K with pH reported here is such that errors in measurement of K are much larger than the small error caused by this assumption. In all cases, the pH values at which association constants are reported are those of the final solution.

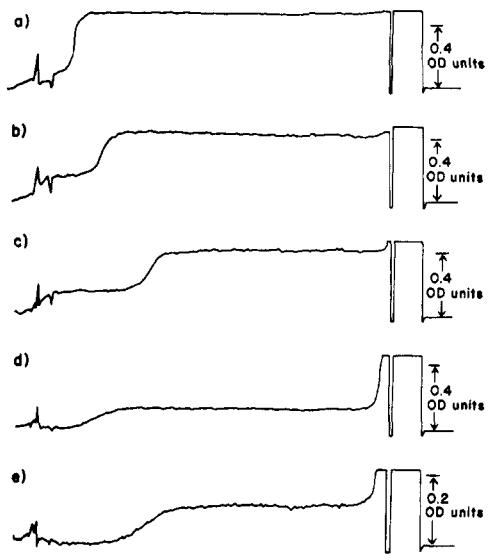

FIGURE 1: Scans from a typical Model E ultracentrifuge experiment on repressor-λ DNA association. Panels a, b, and c show the rapid movement of the heavy DNA-protein complexes; the absorbance adjacent to and just to the right of the meniscus is due to unbound protein. Panels d and e show the slower sedimentation of the free repressor molecules. (a) Scan at λ = 265 nm, 5 min after rotor speed reached 24 000 rpm. (b) Scan at λ = 235 nm, 9 min after rotor speed reached 24 000 rpm. After scan b the rotor speed was changed to 40 000 rpm. (c) Scan at λ = 235 nm, 1 min after rotor speed reached 40 000 rpm. (d) Scan at λ = 235 nm, 45 min after rotor speed reached 40 000 rpm. (e) Scan at λ = 235 nm, 84 min after rotor speed reached 40 000 rpm. Note change of absorbance scale in panel e as compared with panel d. From the height of the plateau region in panel d or e, the concentration of free repressor is evaluated using the optical system calibrations described in the text. Sedimentation is from left to right; the meniscus in the solute sector was at the upward "blip". In this run, the ionic strength was 0.17 M Na^+, pH 7.5, T = 20 °C; input concentrations were $[\lambda\ DNA]_{total}$ = 29.0 × 10^{-6} M base pairs, $[R]_{total}$ = 1.09 × 10^{-6} M (tetramers).

(1970a) and by Lin and Riggs (1972). Equilibrium competition experiments (Lin and Riggs, 1972) were employed to test the relative affinities for binding of repressor to operator and to non-operator-containing DNAs, including native phage λ DNA (purified from wild type phage obtained from Dr. Ira Herskowitz), poly[d(A-T)], and native calf thymus DNA.

Results

The sedimentation procedure described above for measuring repressor-DNA binding constants was devised because the optical changes brought about in the DNA and the repressor as a consequence of the interaction of the components (Matthews et al., 1973; Butler et al., 1977) are too small to be useful for this purpose at the required precision of measurement. Centrifugation separates repressor-DNA complexes from repressor free in solution; the rapid sedimentation of the heavy DNA-protein complexes can be followed (Figure 1), and the concentration of the unbound repressor (R_f) calculated from the absorbance of the slowly sedimenting protein which remains behind. Using this value of R_f and the known input concentrations of repressor and DNA, we can compute the concentration of bound repressor (R_b) and the binding density ($\nu = R_b/(DNA_{total})$, in units of moles repressor tetramer bound per mole DNA base pairs). These data are graphed as Scatchard plots (ν/R_f vs. ν) (Scatchard, 1949; Klotz and Hunston, 1971) and analyzed to extract K and n by the method of McGhee and von Hippel (1974). Each sedimentation run yields one point on the Scatchard plot—a series of such experiments generates the entire binding curve.

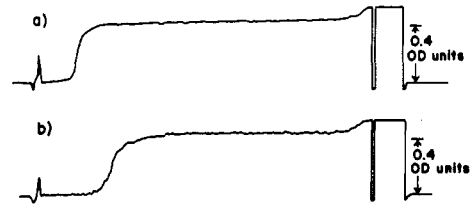

FIGURE 2: Model E ultracentrifuge scans of repressor-DNA solution at low ionic strength showing that all repressor molecules are active in non-specific binding. (a) Scan at λ = 265 nm, 5 min after rotor speed reached 24 000 rpm. (b) Scan at λ = 235 nm, 9 min after rotor speed reached 24 000 rpm. Ionic strength = 0.02 M Na^+, pH 7.8, T = 20 °C. Input concentrations were $[\lambda\ DNA]_{total}$ = 27.3 × 10^{-6} M (base pairs), $[R]_{total}$ = 1.15 × 10^{-6} M (tetramers). Sedimentation is from left to right; the meniscus in the solute sector was at the upward "blip".

Controls, Errors, and Reproducibility. The careful procedures for preparing the sedimenting solutions described in Materials and Methods were designed in part because of the tendency of repressor to aggregate. The fact that these procedures (adjusting the ionic strength of the solution from 0.02 M Na^+ just prior to each experiment) yield equilibrium distributions was demonstrated by comparing sedimentation data obtained on a solution prepared by mixing appropriate amounts of DNA and repressor dialyzed separately against 0.15 M Na^+ buffer with a solution prepared in the way described above. Identical results were obtained in both runs, indicating that equilibrium is indeed attained in these procedures (see also Wang et al., 1977).

Controls for progressive changes in DNA-repressor solutions were also carried out by routinely running aliquots of the same DNA-repressor solution at the beginning and the end of a day of experiments. Identical results were obtained, indicating that solutions were stable over this time period.

To maximize comparability of results, solutions of different ionic strengths were made by adjusting aliquots of the *same* DNA-repressor stocks to the appropriate ionic strengths as described in Materials and Methods. Thus errors made in preparing a given DNA-protein solution are reflected the same way in all the Scatchard plots, and accurate comparative binding parameters are determined.

Effects of Rotor Speed (Pressure Dependence). Many macromolecular interactions result in significant specific volume changes of the components involved (Harrington and Kegeles, 1973); as a consequence observed equilibrium constants may vary enormously with the pressure applied to the system. Since the pressure applied to a solution in an ultracentrifuge cell varies with distance from the axis of rotation and with rotor speed, we have made a series of measurements at different rotor speeds to look for pressure dependence of the repressor-non-operator DNA binding equilibrium. We find that the amount of unbound repressor in equilibrium with the DNA-protein complex is independent of rotor speed at 16 000, 24 000, 32 000, and 40 000 rpm. Figure 1 illustrates this point—note that the absorbance (i.e., concentration) of the slowly sedimenting material (repressor) does not change as rotor speed is increased from 24 000 (Figure 1b) to 40 000 (Figure 1c-e) rpm. Thus the interaction of repressor with non-operator DNA does not appear to bring about a significant change in the specific volume of either component.

All Repressor Molecules Are Active in Non-Operator-DNA Binding. It is apparent from Figure 2, which shows scans of the centrifuge cell at two wavelengths during a sedimentation run at 0.02 M Na^+, that all repressor is active in binding to non-operator DNA. At both 265 and 235 nm all absorbing material sediments as a single boundary; none remains at or

TABLE I: Association Constants for Binding of *lac* Repressor to DNA.

DNA	Temp (°C)	$[Na^+]$[a] (M)	pH	K (M^{-1})[b]	Comments
λ DNA	20	0.13	7.5	11 (±6) × 10^5	Evaluated using data
λ DNA	20	0.15	7.5	2.4 (±0.8) × 10^5	from *all* experiments
λ DNA	20	0.17	7.5	0.7 (±0.2) × 10^5	at the given ionic strength
λ DNA	20	0.15	7.2	12 (±2) × 10^5	
λ DNA	20	0.15	7.5	2.7 (±0.4) × 10^5	Control experiments[c]
λ DNA	20	0.15	7.8	1.4 (±0.2) × 10^5	
λ DNA	10	0.15	7.5	3.8 (±0.8) × 10^5	
λDNA	20	0.15	7.5	2.8 (±0.3) × 10^5	Control experiments[c]
λ DNA	30	0.15	7.5	1.9 (±0.3) × 10^5	
λ DNA	20	0.17	7.5	1.4 (±0.1) × 10^5	Plus 16% glycerol
λ DNA	20	0.17	7.5	0.7 (±0.3) × 10^5	Control (no glycerol)[c]
Poly[d(A-T)]	20	0.15	7.5	14 (±6) × 10^5	Control experiments[c]
λ DNA	20	0.15	7.5	2.2 (±0.1) × 10^5	
Poly[d(A-T)]	20	0.17	7.5	5 (±2) × 10^5	Control experiments[c]
λ DNA	20	0.17	7.5	0.7 (±0.2) × 10^5	

[a] Buffers contained 0.01 M Na_2HPO_4, 0.0001 M Na_2EDTA, plus NaCl to give the desired total Na^+ concentration at the stated pH. [b] Association constants are in units of M^{-1}, with concentrations molar in repressor (tetramer) and DNA base pairs, respectively. [c] Association constants for "control" experiments were derived using data taken in comparative binding studies (see text). For example, at each ionic strength the values of K for repressor–phage λ DNA controls were evaluated from data obtained on the same day as corresponding (paired) data for repressor–poly[d(A-T)] binding.

near the meniscus. For this particular solution approximately 72% of the absorbance at 235 nm is contributed by the protein, so that appreciable levels of free protein should be easily detected. Results similar to those of Figure 2 were obtained (i.e., virtually all the protein remained bound to DNA) when the salt concentration was raised to 0.11 M Na^+. Progressively increasing amounts of unbound protein were then seen in this DNA–repressor solution as the salt concentration was raised further to 0.13, 0.15, 0.17 (Table I), and finally to 0.19 M Na^+. Thus we conclude that the data of Figure 2 reflect tight (equilibrium) binding of *all* the repressor molecules to DNA, and thus that all the protein in the solution is active in non-operator DNA binding.

Determination of Binding Parameters. Scatchard plots of data for repressor–DNA binding under several environmental conditions are shown in Figures 3–5. For large ligands such as repressor, which cover more than one base pair when bound to DNA, Scatchard plots are nonlinear even in the absence of protein–protein binding cooperativity (McGhee and von Hippel, 1974). Our analysis is based on the fact that repressor binding to non-operator DNA is noncooperative.[3]

The exact shape of the binding curve and the positions of the horizontal and vertical intercepts depend on the values of the equilibrium binding constant (K) and the binding site size (n, defined as the number of base pairs covered by a bound repressor molecule). It is straightforward to fit the experimental data to the theoretical equation for noncooperative binding of large ligands to an infinite DNA lattice (McGhee and von Hippel, 1974) using the method of least squares with K and n as adjustable parameters. When this procedure was applied to our data we found that in all cases n lies between 10 and 14 base pairs. This is in good agreement with the more accurate determination of $n \simeq 12$ base pairs obtained from titration experiments at low ionic strength (where essentially all repressor molecules are bound to the DNA) using either circular dichroism or change in repressor sulfhydryl group reactivity to monitor the binding (Butler et al., 1977). Therefore, to increase the accuracy of our comparison of K values, we have set $n = 12$ base pairs in all cases, and fit the data to the theoretical equation using the method of least squares with K as the only adjustable parameter.

Table I summarizes the results obtained for the systems and solvent conditions examined; the values for K in Table I were generated by the above one-parameter approach. As analyzed here, the values of K which best fit the data represent the arithmetic average of the values of K computed for the individual data points.

The binding of repressor to phage λ DNA at 0.15 M Na^+, pH 7.5, T = 20 °C, is shown in Figure 3. The points on this graph were derived from experiments on many different days using different DNA–repressor mixtures; the theoretical curve is calculated assuming $n = 12$ base pairs. Computations using values of n between 10 and 14 base pairs also yield acceptable fits—in fact, the calculated standard deviations are about the same for all these n values. Thus we conclude that for the repressor–phage λ DNA system the sedimentation experiments yield an acceptable value for n, but one which requires confirmation by other techniques (Butler et al., 1977).

From Figure 3 we see also that the association constant can be determined with good accuracy. The calculated value of K is 2.4 (±0.7) × 10^5 M^{-1} from these data (Table I); this value changes from 1.8 × 10^5 to 3.4 × 10^5 M^{-1} as n is varied from 10 to 14 base pairs. Thus regardless of uncertainties in the value of n, we can determine association constants to better than a factor of two by these techniques. We expect *relative* values of K to be even more accurate, since we have performed

[3] All evidence indicates that there are no interactions (other than steric) between adjacent repressor molecules bound to DNA. In the sedimentation experiments reported here we find a single relatively sharp boundary for the DNA–protein complexes and no completely uncomplexed DNA molecules, implying that repressor does not accumulate on some DNA molecules while leaving others devoid of protein as would be expected in a cooperative binding situation (for a further discussion of this point, see Wang et al., 1977). In addition, for systems in which the ligands are large, Scatchard plots are *markedly* nonlinear if the ligands display even a small degree of cooperativity (McGhee and von Hippel, 1974). The experimental data (Figures 3, 4, and 5) strongly indicate that there is, in fact, no cooperativity in the binding of repressor to non-operator DNA and, therefore, that the cooperativity parameter (ω) for this system is $\simeq 1$.

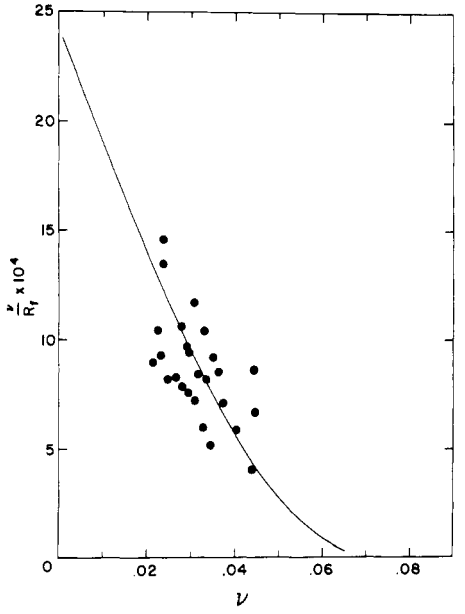

FIGURE 3: Scatchard plot for repressor-phage λ DNA association at 0.15 M Na^+, pH 7.5, T = 20 °C. The theoretical curve was calculated using a binding site size (n) of 12 base pairs, and assuming no protein-protein binding cooperativity (see text).

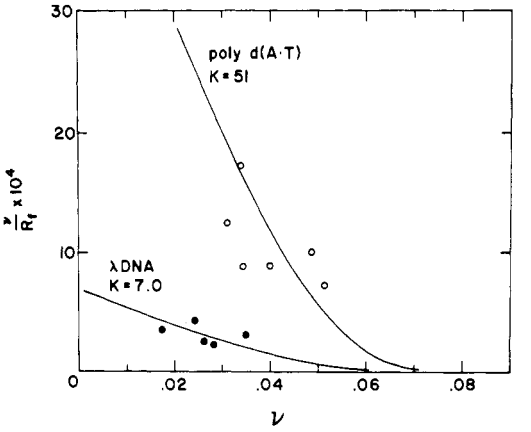

FIGURE 4: Scatchard plots for association of repressor with λ DNA [●] and with poly[d(A-T)] [○], at 0.17 M Na^+, pH 7.5, T = 20 °C. The theoretical curves were calculated using n = 12 and assuming no protein-protein binding cooperativity.

comparison-type experiments wherever possible and analyze all data using the same value (n = 12 base pairs) for the binding site size. The measured (overall) standard deviation of ca. ± 30% for the data in Figure 3 is not unreasonable in light of the difficulties involved in handling repressor (aggregation), and the large number of measurements involved.

Figure 4 shows results obtained at 0.17 M Na^+ for repressor binding to phage λ DNA and to poly[d(A-T)]. We see from this graph that repressor binds ca. sevenfold more tightly to poly[d(A-T)] than to phage λ DNA. In Table I it is shown that at 0.15 M Na^+ the association constant for repressor binding to poly[d(A-T)] is about six times larger than that for binding to phage λ DNA,[4] and similar results have been obtained in thermal melting experiments performed at much lower ionic strengths (0.002 to 0.02 M Na^+; Wang et al. 1977) and equilibrium competition studies using the filter assay (data not shown). All these measurements taken together, involving salt concentrations ranging from 0.002 to 0.17 M Na^+, show that repressor binds poly[d(A-T)] ca. two to seven times more tightly than does phage λ DNA. A similar small preference for poly[d(A-T)] over other competing native DNAs has been demonstrated by the competitive melting technique (Wang et al., 1977).

Effects of Ionic Strength on Binding Constants. Table I shows that the affinity of repressor for phage λ DNA is 3.4 times larger at 0.15 M Na^+ than at 0.17 M Na^+, and that K at 0.13 M Na^+ (although subject to more uncertainty) is about 4.4 times larger than K at 0.15 M Na^+ for this system. From these data we estimate (by extrapolation) that K is $\sim 10^7$ M^{-1} at 0.1 M Na^+, and $\sim 10^4$ M^{-1} at 0.2 M Na^+. This large decrease in K with increasing ionic strength indicates a very substantial electrostatic contribution to the binding energy. Record et al. (1976) have analyzed the effects of ionic strength on DNA-protein interactions, and have shown that in such systems $\delta \log K / \delta \log [M^+] = -m'\psi$; where K is the observed association constant, $[M^+]$ is the concentration of monovalent cation, ψ is the fraction of a counterion thermodynamically bound per nucleic acid phosphate (0.9 for double-helical DNA), and m' is the number of ion pairs involved in the binding of the ligand to DNA. Application of this equation to our data yields a value of approximately 11 (±1) ion pairs formed when a repressor molecule binds nonspecifically to phage λ DNA or to poly[d(A-T)], assuming no significant anion binding to repressor (see Record et al., 1976).[5]

Effects of Mg^{2+} Concentration. We have been unable to determine accurate binding constants for repressor to DNA in the presence of Mg^{2+} ions, because the solutes displayed a marked (if somewhat erratic) tendency to aggregate. However, enough experiments were successful (i.e., free of aggregates) to permit some qualitative conclusions to be drawn. In particular, we observe that all the protein remains bound to the DNA in a repressor-DNA solution at 0.01 Mg^{2+} (in 0.02 M Tris, pH 7.8), while at 0.025 M or 0.05 M Mg^{2+} in the same buffer virtually all the protein is free in solution.

In a buffer containing 0.025 M Mg^{2+} and 0.02 M Tris, the ionic strength is 0.12 (if all Tris cations contribute fully). In our other experiments we observe that at this ionic strength, in the presence of only monovalent cations, there will be a substantial amount of repressor bound to DNA even at pH 7.8. Therefore the effect of Mg^{2+} on the repressor-DNA interaction is more than a simple ionic strength effect. That is, in terms of ion concentrations, Mg^{2+} is more than four times better than Na^+ in weakening the binding. On the other hand, the effect of Mg^{2+} on repressor binding is not as pronounced as one might expect from comparison of divalent to monovalent cation effects on certain other aspects of polyelectrolyte behavior (e.g., the effect of Mg^{2+} concentration on DNA melting temperatures; Dove and Davidson, 1962); see also deHaseth et al. (1977b).

Effects of pH. The effects of pH on the repressor-DNA association constant are shown in Figure 5 and in Table I. It

[4] The uncertainties in K for the interaction of repressor with poly[d(A-T)] are relatively high. This arises because the values of the association constants are larger. As a result, at the concentrations required to perform experiments in the Model E, there is rather little free repressor in the system and a small error in measurement of R_f leads to a magnified uncertainty in the quantity ν/R_f. Nevertheless, based on experiments performed on a given day comparing the binding of repressor to phage λ DNA and to poly[d(A-T)] (see paired data sets in Table I), there is no doubt that the protein binds about six times more tightly to the latter than to the former.

[5] Recently, using a completely different DNA-cellulose chromatography technique (deHaseth et al., 1977a), Record and co-workers have also obtained a value of m' = 12 (±2) ion pairs for the repressor-nonspecific DNA interaction (deHaseth et al., 1977b).

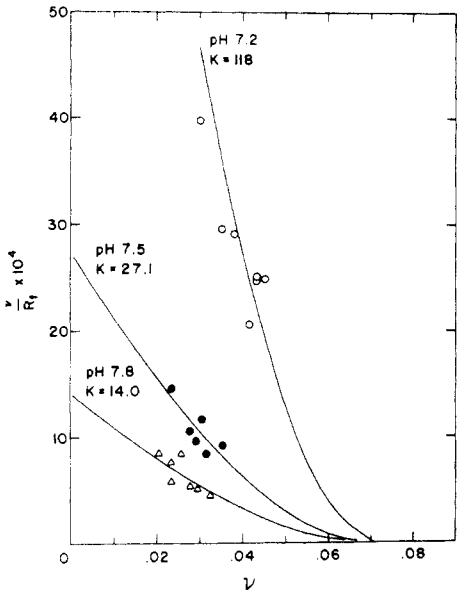

FIGURE 5: Scatchard plots for association of repressor with λ DNA at pH 7.2 (○), pH 7.5 (●) [control experiments], and pH 7.8 (△); T = 20 °C, 0.15 M Na^+. The theoretical curves were calculated using n = 12 and assuming no protein-protein binding cooperativity.

is clear that K decreases with increasing pH in the range pH 7.2 to 7.8. This indicates (but certainly does not prove) that among the amino acid side chains in close contact with the DNA is a histidine residue (or residues); if a positively charged histidine does indeed make an electrostatic contribution to the binding energy, then we expect a decrease in affinity at higher pH as the residue becomes uncharged. We note that the N-terminal peptides of repressor, which are primarily involved in DNA binding, do indeed contain a histidine residue (Beyreuther et al., 1973; Platt et al., 1973).[6]

Effects of Temperature. Repressor-phage λ DNA binding curves were also measured at temperatures of 10, 20, and 30 °C (at pH 7.5, 0.15 M Na^+), in order to determine the effects of this variable upon the binding affinity; association constants determined under these conditions are also listed in Table I. These results permit us to estimate thermodynamic parameters for the reaction. From a van't Hoff plot we find $\Delta H^\circ = -6.0$ kcal/mol. At 20 °C, $\Delta G^\circ = -7.3$ kcal/mol and therefore $\Delta S^\circ = 4.4$ eu for the repressor-non-operator DNA interaction.

Effects of Inducer Binding. We previously reported preliminary results from glycerol gradient experiments which implied that repressor-inducer complexes bind to non-operator DNA with the same affinity as does repressor alone (von Hippel et al., 1975). We have confirmed and extended these data using the Model E sedimentation technique. In these experiments, two 0.4-mL aliquots of a DNA-repressor solution were run consecutively in the centrifuge. The aliquots differed only in that 4 μL of 0.1 M IPTG had been added to one (thus giving an inducer concentration of 10^{-3} M) while 4 μL of buffer was added to the other. Experiments were performed at different ionic strengths (at pH 7.5) and at different pH values (at 0.15 M Na^+). In all cases, the pairs of runs gave identical results. Thus binding of inducer to repressor does not affect the binding of repressor to DNA under the solvent conditions studied, nor does DNA binding alter the affinity or stoichiometry of inducer binding by repressor (see also Butler et al., 1977; Wang et al., 1977).

Binding of Trypsin-Treated Repressor to DNA. It has been shown that the primary effect of limited trypsin digestion of repressor is the cleaving of an ~58 residue polypeptide fragment from the N-terminal end of each monomer subunit, leaving intact a more trypsin resistant tetrameric "core" of protein (Platt et al., 1973). We previously reported experiments in glycerol gradients which indicated a much reduced affinity of "core" repressor for DNA, as compared with binding of intact repressor (von Hippel et al., 1975). These results were confirmed by the Model E technique, using "core" fragments prepared and kindly provided by Dr. Andrew P. Butler. We could detect no binding of "core" repressor to phage λ DNA in either 0.15 M Na^+ or 0.02 M Na^+ (T = 20 °C, pH 7.5). This means that, even at low ionic strength, where K for the unmodified repressor-DNA reaction is greater than 10^9 M^{-1}, the association constant for "core" repressor to DNA is no greater than about 10^4 M^{-1}, and could be much smaller.

Effects of Glycerol on Repressor-DNA Associations. Use of the Model E ultracentrifuge permits evaluation of binding parameters in the absence of the relatively high concentrations of glycerol or sucrose required in sedimentation experiments using gradients. This is desirable since both glycerol and sucrose do somewhat destabilize the DNA double helix (Levine et al., 1963; Marmur et al., 1963), and hence could perturb protein-DNA interactions. The binding of repressor to phage λ DNA at 0.17 M Na^+ (pH 7.5, T = 20 °C), in solutions containing 16% glycerol and no glycerol, is compared in Table I; the association constant is elevated ca. twofold in the glycerol-containing solution.

It has been reported that glycerol can activate certain promoters (Nakanishi et al., 1974; Crepin et al., 1975). These workers have suggested that glycerol may act by lowering the stability of the DNA double-helix at the promoter region. Our data show a (small) stabilizing effect of glycerol on the non-operator DNA binding of *lac* repressor. Insofar as these results are applicable to the control region itself, glycerol-induced melting at the promoter must be sufficient to overcome the stabilization of DNA-repressor binding, though the observed increased repressor binding at non-operator DNA loci could reduce the free repressor concentration and thus offset this effect (von Hippel et al., 1974). Alternatively, glycerol could have different (specific) effects on either the repressor-operator or the polymerase-promoter interaction.

Discussion

Comparison of Sedimentation Techniques for Measuring Binding Parameters. The techniques for measuring binding parameters described here and in Jensen and von Hippel (1977) should yield absolute equilibrium binding parameters, since many of the complications of (e.g.) band sedimentation experiments are avoided by having both components initially distributed uniformly throughout the solution. Controls and assumptions applying to both the preparative gradient centrifugation (Jensen and von Hippel, 1977) and the analytical centrifugation (this work) techniques have been discussed elsewhere (Jensen and von Hippel, 1977). In this section we compare the two approaches.

In the analytical (optical) method the solvent can be better controlled, in that extraneous density gradient forming materials need not be added. Also fraction collection problems and gradient artifacts are avoided, aggregation problems are im-

[6] Using comparable DNA-cellulose chromatography data obtained at various values of pH, de Haseth et al. (1977b) have shown that *two* groups per repressor molecule must be protonated in binding to nonspecific DNA. It is reasonable to assume that these groups are histidine residues, and since the DNA-binding N-terminal sequences of the *lac* repressor subunits contain only one histidine residue each (residue 29), this result also implicates *two* repressor subunits in the DNA interaction (see also Butler et al., 1977).

mediately detected, and the experiment can be evaluated and data collected and processed *during* the run. The main disadvantage of the analytical ultracentrifuge technique is that the concentration range amenable to study is limited by the absorbance-measuring capabilities of the UV scanner. It is for this reason that our data span such a narrow ionic strength range, and must be extended by extrapolation.

The preparative gradient ultracentrifugation approach also has advantages. Comparison of repressor–DNA association in glycerol-containing and glycerol-free solutions implies that, for this system, the perturbation of the binding due to the presence of gradient forming materials is not large. And the major advantage of gradient studies is that fractions can be analyzed for protein or DNA by a variety of techniques, including intrinsic protein fluorescence, radioactive labeling of either DNA or protein, absorbance, etc. This greatly increases the potential concentration range over which binding constants can be measured.

Binding of Repressor to Operator and to Non-Operator-Containing DNA. The characteristics of repressor binding to non-operator-containing DNA may be compared with what is known about the specific association of repressor with operator. It has been previously shown that repressor binds preferentially to double-helical DNA in both the specific and nonspecific binding modes (Riggs et al., 1970b; Lin and Riggs, 1972; von Hippel et al., 1975). Studies using a thermal melting technique indicate that the affinity of repressor for single-stranded DNA is at least 50-fold weaker than that for native DNA (Wang et al., 1977). Since double-stranded DNA is also the predominant species present in vivo, we have focused on repressor binding to native DNA.

The magnitudes of the repressor association constants for operator and non-operator DNA binding are, of course, very different. We find values for K of about 10^5 M^{-1} for nonspecific DNA binding to 0.15 M Na^+, while under comparable ionic conditions the association with operator is tighter by seven to nine orders of magnitude (Riggs et al., 1970a,b).

It is known that the binding of inducer to repressor reduces the affinity of the protein for the operator region by about three orders of magnitude (Jobe and Bourgeois, 1972). Whatever the conformational changes involved in this process, they apparently play no role in non-operator DNA binding since we find no effect of inducer binding on the association constant of repressor with non-operator DNA under a variety of solvent conditions. This fact was inferred by Lin and Riggs (1972) using indirect evidence from filter assay experiments; we have here confirmed, by direct binding measurements, that IPTG does not affect the repressor–non-operator DNA binding equilibrium.

The results presented here confirm our findings with other techniques that about 12 base pairs are covered when repressor binds to non-operator-containing DNA (Butler et al., 1977), assuming binding to one "side" of the DNA lattice only. This contrasts with the observation that repressor which is specifically bound to the operator region protects about 25 base pairs from nuclease digestion (Gilbert and Maxam, 1973). Possible rationales for these differences in apparent repressor binding site size have been discussed in Butler et al. (1977).

There are a number of striking similarities between operator and non-operator binding of repressor to DNA. The inducer binding site on the protein is accessible whether repressor is bound to the operator or to non-operator regions. Furthermore it is clear that the 58-residue N-terminal fragment cleaved from repressor subunits by limited tryptic digestion is absolutely essential for both types of binding. This is consistent with the fact that the repressor–non-operator DNA interaction involves a large electrostatic component, and with the fact that, while the repressor as a whole is negatively charged at neutral pH, the N-terminal peptide contains one histidine, three arginine, and four lysine residues, and carries a substantial net positive charge (Beyreuther et al., 1973; Platt et al., 1973). Applying the theory of Record et al. (1976) to our data on the ionic strength dependence of the binding constant we arrive at a value of ~11 ion pairs formed per bound repressor tetramer; this is compatible with amino acid composition data since each tetramer contains ~30 positively charged amino acid residues in N-terminal sequences which may be available to interact with DNA phosphates. (We note that one repressor subunit can form a maximum of 8 ion pairs on this basis, while two subunits can form as many as 16; again suggesting the involvement of more than one repressor subunit in the DNA interaction.)

The ionic strength dependences of binding of repressor to operator and non-operator DNA are also similar (Lin and Riggs, 1975); we find $\delta \log K/\delta \log [Na^+] \simeq -10$ for nonspecific binding in the monovalent ion concentration range studied. Interpretation of filter assay data for the ionic strength dependence of repressor–operator binding is complicated by the presence of Mg^{2+} in the binding buffer (Riggs et al., 1970a,b). However, Record et al. (1977) have recently performed calculations on these data which take into account the effect of the Mg^{2+} ions and yield $\delta \log K/\delta \log [K^+] \simeq -7$ for repressor–operator binding, suggesting that ~8 ion pairs are involved in the repressor–operator interaction.

The temperature dependence of the repressor–operator association has been studied by Riggs et al. (1970b). They estimated the enthalpy change on binding ($\Delta H°$) to be ~+8.5 kcal/mol, and $\Delta S°$ for the binding reaction to be +90 cal mol^{-1} deg^{-1}. Based on these results Lin and Riggs (1972), using competitive filter binding procedures, concluded that there should be little effect of temperature on the binding of repressor to non-operator DNA. Our data are in reasonable accord with this conclusion, yielding the following values for the thermodynamic parameters for binding of repressor to non-operator DNA: $\Delta H° = -6$ kcal/mol and $\Delta S° = +4.4$ cal mol^{-1} deg^{-1}. These results indicate a much smaller entropic effect for non-operator DNA binding than for the repressor–operator interaction. This might be expected if repressor binding to operator involves intimate (and water molecule displacing) associations between repressor and base-specific functional groups located in the grooves of the DNA molecule (as well as a conformational change in the repressor), while non-operator binding involves primarily an electrostatic interaction between positively charged repressor residues and the negatively charged phosphates of the DNA sugar–phosphate backbones.

In this paper we have presented some limited studies on the base composition and sequence dependence of repressor binding to non-operator DNA. Lin and Riggs (1972), using the competition filter assay method, reported that repressor shows a marked preference for poly[d(A-T)] in comparison with other DNAs. For example, they estimated the binding to be about 25 times tighter to poly[d(A-T)] than to phage λ DNA. Further studies indicated a preference for certain other synthetic polymers containing repeating trimer and tetramer base sequences (Riggs et al., 1972). In our experiments we find a somewhat smaller poly[d(A-T)] preference, repressor binding only six to seven times more tightly to poly[d(A-T)] than to phage λ DNA. This result has been confirmed using a thermal melting approach (Wang et al., 1977). Furthermore, in our hands the filter assay also shows a relatively small preference of repressor for poly[d(A-T)]. Preliminary exper-

iments indicate that the molecular weight of the competing DNA may appreciably influence its effectiveness as a competitor in filter binding experiments (R. B. Winter and P. H. von Hippel, in preparation). This observation may largely explain the discrepancies between our absolute measurements of repressor–non-operator DNA binding constants, and those inferred by others from competition filter binding assays.

Physiological Implications. Finally, we consider the implications of these results for a possible in vivo regulatory role for non-specific DNA binding of genome control proteins (von Hippel et al., 1974; Chamberlin, 1974; Lin and Riggs, 1975). While the association of repressor with non-operator DNA is much weaker than is specific binding, there exist a very large number of potential non-specific DNA binding sites on the bacterial chromosome. Thus while there is only one lactose operator region per genome, every base pair on the chromosome represents a potential non-specific binding site (McGhee and von Hippel, 1974). In a theoretical analysis (von Hippel et al., 1974), we estimated that values of $K_{RD} > 10^2\ M^{-1}$ for non-specific binding may cause repressor molecules not bound to the operator to be found primarily in association with other DNA sequences, and indeed that such non-specific DNA binding may be essential if the operon is to be inducible in the presence of lactose. Pfahl (1976) and Betz and Sadler (1976) have invoked non-specific binding to interpret certain physiological characteristics of *E. coli* containing mutant repressors with enhanced affinities for the operator.

Our non-specific association constants were evaluated at $[Na^+] \simeq 0.15$ M, in the absence of divalent ions. We have shown that the presence of Mg^{2+} ions decreases the affinity of repressor for DNA; while there are, of course, divalent ions within the bacterium, a large fraction of these will be sequestered in various ways (e.g., Mg^{2+} ions may be bound to ATP) and thus will not be available to compete with repressor for binding to the chromosome. Therefore a value of $K_{RD} \simeq 10^5\ M^{-1}$ may indeed represent a reasonable approximation to the in vivo value. We have recently performed experiments using "minicells" of *E. coli* (Adler et al., 1967) which show that virtually all the repressor molecules are indeed associated with the *E. coli* chromosome in vivo (Kao- Huang et al., 1977).

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