Biochemistry

 Volume 16, Number 22 November 1, 1977

Molecular Parameters Characterizing the Interaction of *Escherichia coli lac* Repressor with Non-Operator DNA and Inducer†

Andrew P. Butler,‡ Arnold Revzin,§ and Peter H. von Hippel*

ABSTRACT: The stoichiometries of binding of non-operator DNA and inducer to *lac* repressor, as well as some conformational aspects of these interactions, are described in this paper. It is shown that the circular dichroism (CD) spectrum of the repressor–non-operator DNA complex is appreciably different from that obtained by summing the spectra of the separate components; the major change is a substantial enhancement of the positive (~275 nm) lobe of the originally conservative DNA B form pattern. These CD spectral changes appear to reflect a change in DNA conformation on repressor binding and are interpreted in terms of tilting (relative to the DNA axis) of some of the base pairs of the native structure, or perhaps some twisting of the overall structure resulting in a tighter coupling of vicinal base transition moments. These changes in the CD spectrum of non-operator DNA on repressor binding have been monitored as a function of added repressor concentration, under tight-binding conditions, to establish that the site size (n) for binding to non-operator DNA is ~12 base pairs per repressor tetramer (~24 base pairs if repressor binds to *both* sides of the double helical DNA lattice). This value of n is confirmed both by calculation from binding isotherms (Revzin, A., and von Hippel, P. H. (1977), *Biochemistry 16* (following paper in this issue), and by titration of repressor sulfhydryl groups as a function of added DNA concentration. The measured site size is discussed in terms of the relationship between operator and non-operator DNA binding of repressor, and of various features of the known operator sequence, to suggest alternative models for the geometry of the repressor–operator interaction. Investigation of the repressor–inducer interaction by equilibrium dialysis, fluorescence, and gel permeation chromatography shows, in confirmation of the results of Ohshima, Y., et al. ((1974) *J. Mol. Biol. 89*, 127), that different repressor preparations exhibit different (average) numbers of "active" inducer binding sites per repressor tetramer (n_I); values of n_I between two and four have been obtained. The effects on n_I of a variety of environmental conditions have been examined, and the results, together with relevant data from the literature, are discussed in terms of conformational equilibria between forms of repressor subunits which bind strongly to inducer and weakly to operator (R_I), and forms which bind strongly to operator and weakly to inducer (R_O). Thermodynamic parameters for the binding of inducer to repressor subunits (in the R_I form) have also been determined. At pH 7.6 (4–25 °C), $\Delta G° = -7.7$ kcal/mol (25 °C), $\Delta H° = -6.2$ kcal/mol, and $\Delta S° = +5$ cal mol^{-1} deg^{-1} for this reaction.

The *lac* repressor recognizes (and binds tightly to) a particular sequence of base pairs in the *Escherichia coli* chromosome which is defined, on this basis, as the *lac* operator. By virtue of this specific binding the repressor exerts negative control over the expression of the lactose operon (Jacob and Monod, 1961). The interaction of *lac* repressor with operator has been subjected to intense scrutiny by both biochemical and genetic means, and a great deal of information is now available about both the protein and the operator (for a recent review, see Bourgeois and Pfahl, 1976).

To a first approximation, DNA must present an overwhelmingly monotonic structural facade to a DNA-binding protein seeking a particular double-helical sequence, since in terms of potential interacting groups the structure is dominated by the highly charged and hydrophilic sugar–phosphate backbones. The presumptive major sources of interaction specificity—the functional groups identifying individual base pairs which are exposed via the large and small grooves of the overall structure—appear distinctly secondary by comparison. The interaction of *lac* repressor with operator DNA is highly ionic strength dependent (Lin and Riggs, 1972, 1975a). This indicates that ion-pair bonds between positively charged protein residues and negatively charged phosphates of the operator sequence do contribute significantly to the stability of the complex (Record et al., 1976), though perhaps not to its specificity (for a general review of DNA–protein interaction mechanisms, see von Hippel and McGhee, 1972). These observations suggest that repressor should exhibit an appreciable

† From the Institute of Molecular Biology and the Department of Chemistry at the University of Oregon, Eugene, Oregon 97403. *Received March 11, 1977.* The research reported here was supported largely by U.S. Public Health Service Grants GM-15792 (to P.H.v.H.) and GM-15423. A.R. was the recipient of a U.S. Public Health Service postdoctoral fellowship (GM-55928) and A.P.B. was a predoctoral trainee on U.S. Public Health Service Grant GM-00715. Some of the research described in this paper was performed (by A.R.) in East Lansing and supported by National Science Foundation Research Grant PCM75-23696.

‡ Present address: Division of Biology, Oak Ridge National Laboratory, Oak Ridge, Tenn. 37830.

§ Present address: Department of Biochemistry, Michigan State University, East Lansing, Mich. 48824.

affinity for *non-operator* DNA (i.e., non-specific binding), and such binding was demonstrated some years ago by Lin and Riggs (1972) using competitive filter binding assay techniques.

It has become clear more recently that this non-specific binding could be of considerable physiological significance in determining, via coupled equilibria, the chemical potential (effective concentration) of free repressor in the cell. It appears that non-specific binding is a crucial component in controlling the binding equilibria of (uninduced and induced) repressor with operator DNA in vitro, and could well play a key role in controlling the level of repression of the operon in vivo (von Hippel et al., 1974, 1975; Lin and Riggs, 1975b; Kao-Huang et al., 1977).

In addition, non-specific binding doubtless explains the apparently anomalous kinetic observation that *lac* (and λ) repressor seems to arrive at the target operator in in vitro filter-binding experiments at rates which appear faster than theoretically possible (i.e., faster than diffusion controlled: Lin and Riggs, 1972; von Hippel and McGhee, 1972; Richter and Eigen, 1974; von Hippel et al., 1975). Thus such binding may also play an important role in the in vivo translocation of DNA-binding proteins.

Finally it has seemed eminently reasonable to many workers in the field that studies of the binding of repressor to non-operator sequences of synthetic or natural DNA should provide considerable insight into the molecular elements of the specific interaction between repressor and operator (Lin and Riggs, 1972; Riggs et al., 1972; von Hippel et al., 1975; Richmond and Steitz, 1976).

In order to provide a proper molecular foundation for the elucidation of the various features of non-specific binding, it is necessary to have detailed information on binding parameters of *lac* repressor to non-operator DNA as a function of environmental conditions. In this and the accompanying paper (Revzin and von Hippel, 1977), we report direct physical chemical measurements of these parameters.

In addition, the interaction of repressor with inducer, resulting in the allosteric conversion of the former to a conformation which binds more weakly to the operator, is also of considerable importance in the overall skein of coupled equilibria between repressor, inducer, operator, and non-operator DNA which ultimately controls the level of repression of the *lac* operon (e.g., see von Hippel et al., 1974). Some aspects of these interactions are understood (see Bourgeois and Pfahl, 1976), but little is known about the structural details of the conformational interconversions between free and inducer-bound repressor. Physicochemical data which further define some aspects of these problems are also presented here.

Preliminary reports of some of the work summarized in these papers have been presented previously (Revzin et al., 1974; von Hippel et al., 1975; Butler et al., 1976).

Materials and Methods

Chemicals and Nucleic Acids. All chemicals used were reagent or spectral grade. Ultrapure guanidinium chloride was obtained from Schwarz/Mann. Unlabeled isopropyl β-D-thiogalactoside (IPTG)[1] was purchased from either Schwarz/Mann or Aldrich, and ^{14}C-labeled IPTG from either Schwarz/Mann or Calatomic. The purity of each IPTG preparation was determined by proton magnetic resonance, and by thin-layer chromatography (TLC) on both cellulose and silica sheets. Labeled and unlabeled IPTG comigrated in all the TLC systems used. We obtained 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) from Sigma and 4,4′-dithiodipyridine (DTP) from Aldrich. The preparation and characterization of the nucleic acids and polynucleotides used are summarized in the accompanying paper (Revzin and von Hippel, 1977).

Purification of lac Repressor. Repressor was isolated, by procedures described previously (Laiken et al., 1972), from *E. coli* (K12) strains containing a temperature-inducible λ lysogen carrying the *lac* genes. We used the BMH 461 and the CSH 46 (M96) repressor "over-producing" strains, carrying presumed promoter mutations i^Q (quantity) and i^{SQ} (super-quantity) in the repressor (i gene) promoter, respectively. Purification involved ammonium sulfate fractionation followed by chromatography on phosphocellulose columns (Laiken et al., 1972); IPTG-binding fractions eluted from the phosphocellulose column were reprecipitated with ammonium sulfate, dissolved in "storage buffer" [1 M Tris-HCl, 3×10^{-4} M EDTA, 30% (v/v) glycerol; pH 7.6 at 25 °C], and stored as small aliquots at −70 °C. The purity of each batch of repressor was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Most preparations were also monitored for operator-binding activity (and IPTG-induced release) by standard filter binding procedures and were shown to be active at levels characteristic of native repressor preparations as defined by this assay (Riggs et al., 1970).

Gel Electrophoresis of Repressor. The protein was denatured and dissociated into single polypeptide chain monomers in 1% sodium dodecyl sulfate solution by heating at 100 °C for 5 min. The gels used were 5-mm diameter Biophore precast 7.5% polyacrylamide gels (Bio-Rad Laboratories), and the reservoir buffer was 0.2 M Tris-base, 0.2 M acetic acid, 0.1% sodium dodecyl sulfate. About 10–20 μL of protein solution (containing 10–40 μg of repressor) was layered on top of each gel; total time of electrophoresis was about 5 h at 3 mA per tube. Gels were stained in a solution of 0.1% Coomassie brilliant blue in 50% methanol, 10% acetic acid, 40% H_2O for 12 h. Destaining buffer was 6% methanol, 9% acetic acid, 0.07 M NaCl. Relative concentrations of contaminants were determined quantitatively by peak area measurements of gel scans at λ = 550 nm, using a Gilford recording spectrophotometer with gel-scanning attachment.

Determination of the Extinction Coefficient of lac Repressor. To establish correct DNA- or inducer-binding stoichiometries for *lac* repressor, it is crucial to have accurate measurements of repressor concentration. Ultraviolet absorbance at 280 nm has generally been used for this purpose, since repressor contains appreciable numbers of aromatic residues. The original literature value of the extinction coefficient (ϵ_{280}) of repressor (Müller-Hill, 1971), used by us and others in earlier reports, appears to be appreciably in error. We therefore redetermined this parameter by three independent means. The absorbance at 340 nm (A_{340}) of all the repressor solutions used in these experiments was less than 2% of the A_{280} value, indicating that the repressor is essentially unaggregated.

Two spectroscopic methods were used, patterned after procedures developed by Edelhoch (1967). Native repressor (>90% pure) was dissolved in 0.1 M NaCl, 0.01 M Tris, 10^{-4} M Na_2EDTA, pH 7.6 (at 25 °C). In one approach an aliquot of stock solution was made 6 M in guanidinium chloride (pH 7.5) and the absorbance of the denatured repressor was measured at 280 nm and 288 nm. This permits calculation of the

[1] Abbreviations used: GdmCl, guanidinium chloride; Tris, tris(hydroxymethyl)aminomethane; Na_2EDTA, disodium ethylenediaminetetracetate; DTP, 4,4′-dithiodipyridine; IPTG, isopropyl β-D-thiogalactoside; CD, circular dichroism; TLC, thin-layer chromatography; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); UV, ultraviolet; PPO, 2,5-diphenyloxazole.

TABLE I: Extinction Coefficient of Native *lac* Repressor at 25 °C.

Method of protein concn determination	ϵ_{280} (M^{-1} cm^{-1})[a]	No. of determinations
Spectral measurement in neutral M GdmCl	2.19 (±0.24) × 10^4	6
Spectral alkaline tyrosine titration	2.35 (±0.14) × 10^4	4
Amino acid analysis	2.25 (±0.08) × 10^4	3
Mean value	2.25 (±0.16) × 10^4	

[a] ϵ_{280} is based on repressor concentrations expressed in units of moles of subunits (monomers) per liter.

concentrations of tyrosine and tryptophan residues. A second spectral method involves comparison of the absorbance spectra of guanidine-denatured repressor at pH 7.5 and pH 12.5. Since the observed spectral changes are due solely to tyrosine ionization, this gives an independent determination of the tyrosine residue concentration. The results of either of these optical measurements, coupled with a knowledge of the number of tyrosine (8) or tryptophan (2) residues per repressor subunit (Platt et al., 1973; Beyreuther et al., 1973), yield the protein concentration in the stock solution. From this we calculate the extinction coefficient of native repressor (ϵ_{280}, M^{-1} cm^{-1}).

A third method is based on the known amino acid composition of *lac* repressor (Platt et al., 1973; Beyreuther et al., 1973). The absorbance of a concentrated solution of repressor in a low salt buffer (0.02 M KCl, 0.01 M Tris, pH 7.6) was recorded, and aliquots were lyophilized and then hydrolyzed in 6 M HCl at 106 °C for periods of 24, 48, and 72 h. Known amounts of norleucine were added to each hydrolyzed sample as an internal standard to correct for loss during transfer to the columns of the Technicon Model TSM amino acid analyzer. Where necessary, the results were corrected for residue destruction during acid hydrolysis by back-extrapolation to zero hydrolysis time. The concentrations of the nine best resolved amino acid residues eluted from the "long" column of the analyzer were used to calculate repressor concentrations for extinction coefficient determinations.

In Table I we summarize values for the extinction coefficient, together with the method used to establish the protein concentration in each set of experiments. The most precise results are those based on amino acid analyses, but all three methods agree within experimental uncertainty. Measurements were made on both i^Q and i^{SQ} repressor preparations; as expected the extinction coefficients determined for both are identical within experimental error.

All repressor concentrations reported in this and the following paper (Revzin and von Hippel, 1977) are based on the average of the extinction coefficients listed in Table I; a value $\epsilon_{280} = 2.25 \times 10^4$ M^{-1} cm^{-1} (*per mole of repressor subunit*) has been used throughout. Values of ϵ_{280} close to this have also recently been determined by others: Huston et al. (1974), and Clement and Duane (1975), based on index of refraction measurements; M. D. Barkley (unpublished, cited in Bourgeois and Pfahl, 1976), based on Lowry analysis of protein concentration; and C. Hélène (personal communication), based on Kjeldahl nitrogen and amino acid composition.

We note that estimation of ϵ_{280} on the basis of the tryptophan and tyrosine content of repressor, using extinction coefficients appropriate to these residues in unstructured peptides (Mihalyi, 1968), yields an extinction coefficient *for the completely denatured protein* of 2.07 × 10^4 M^{-1} cm^{-1}. Thus the absorbance of native repressor at 280 nm is approximately 8% hyperchromic, a result well within the usual 0 to 20% hyperchromicity range observed for aromatic residues in globular proteins (Beaven and Holiday, 1952).

Sedimentation Equilibrium. Repressor molecular weights were measured by standard short-column sedimentation equilibrium procedures, using a Model E Spinco analytical ultracentrifuge equipped with an electronic speed control and absorption optics monitored by a photoelectric scanner system. Repressor concentrations were determined at various positions in the cell by measuring absorbances at 280 nm against a solvent blank. The sample sector of the aluminum-epon 12-mm-path-length double-sector cell contained 0.05 mL of FC-43 fluorocarbon oil (to provide an appropriately shaped and positioned cell base), and ~0.12 mL of repressor solution which had been dialyzed against 0.2 M KCl, 0.01 M Tris, 10^{-4} M Na_2EDTA (pH 7.74) at 22 °C. This resulted in a sample column ~3–4 mm long. The reference sector contained ~0.20 mL of dialysate. The rotor speed was 7200 rpm, and the temperature was maintained at 22.4 °C. An An-J rotor was used and 24 to 36 h was required to attain equilibrium.

Circular Dichroism. Circular dichroism titrations of nonoperator DNA with *lac* repressor were performed on a Cary 60 CD apparatus, using 10-mm path length cells. Titrations were generally made by adding concentrated aliquots of DNA to a repressor solution in the CD cell. After each addition, the absorbance spectrum of the solution was read using a Cary 15 UV spectrophotometer, and the CD spectrum recorded by scanning through the wavelength range several times. Data were accumulated in a Varian 620-L computer and were averaged, base-line corrected, and plotted using the computer to drive a Houston Instruments Omnigraphic X-Y recorder.

Measurement of IPTG Binding to lac Repressor. By Equilibrium Dialysis. Dialysis bags (Union Carbide) were pretreated by boiling several times in fresh 5% $NaHCO_3$ containing 10^{-3} M Na_2EDTA. An aliquot of IPTG-free repressor was placed in each bag and dialyzed against buffer (see figure legends) containing [^{14}C]IPTG and a known amount of unlabeled IPTG. The samples were shaken on mechanical agitators in controlled temperature rooms for 20 to 24 h. Controls showed that dialysis equilibrium was attained in 16 h or less.

After equilibration, the contents of each bag were removed and the concentration of repressor was determined by measuring the absorbance at 280 nm (correcting for any scattered light measured at 340 nm). IPTG concentrations inside and outside the bags were determined by scintillation counting of appropriate aliquots of the repressor solutions and dialysates using a toluene-ethanol-2,5-diphenyloxazole (PPO) scintillation "cocktail".

By Fluorescence Titration. The intrinsic tryptophan fluorescence of repressor was excited at 295 or 310 nm, and the extent of inducer binding was followed by monitoring the resulting decrease in fluorescence intensity at 360 or 370 nm with a Hitachi MPF-2A spectrofluorimeter (Laiken et al., 1972). A 310 or 350 nm cut-off filter was used to ensure that no scattered exciting light reached the emission monochromator. All titrations were performed at 25 °C.

By Gel Permeation Chromatography. Inducer binding to repressor was also measured by the method of Hummel and Dreyer (1962). A 1 × 46 cm column containing Bio-Gel P-6 was equilibrated with buffer containing [^{14}C]IPTG and a fixed amount of unlabeled IPTG. (This establishes the free ligand level of IPTG.) Aliquots of solution containing repressor and IPTG (concentrations of the latter ranged from zero to well above that of the column equilibrating buffer) were layered on the column, which was then eluted with the IPTG-con-

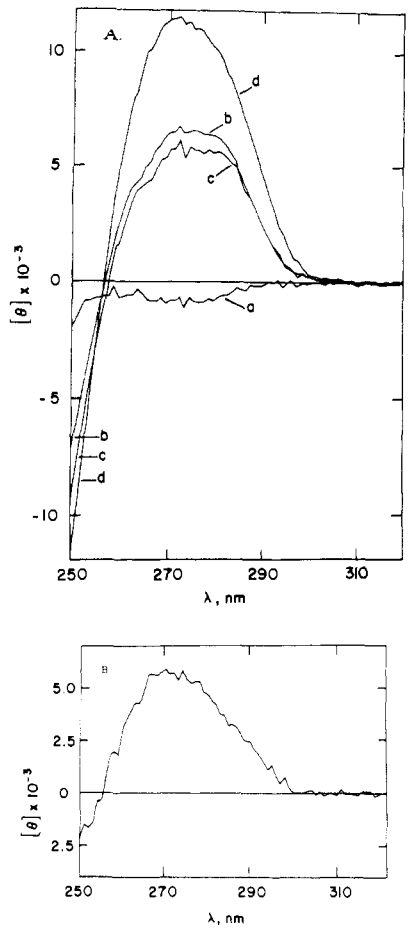

FIGURE 1: (A) Circular dichroism spectra of *lac* repressor and calf thymus DNA. Spectrum a is for repressor alone; b represents calf thymus DNA alone; c is the sum of a and b, assuming no interaction; and d is the *observed* repressor plus DNA spectrum at a binding ratio of 18 base pairs per tetramer. The spectra are expressed in units of molar ellipticity (per mole DNA phosphate). Curve a is the spectrum for repressor at a concentration corresponding to that in curve d. Buffer: 0.02 M Tris, 10^{-4} M Na_2EDTA, pH 7.5; temperature = 27 °C. Under these conditions virtually all the repressor molecules are bound to the DNA (Revzin and von Hippel, 1977). (B) Difference circular dichroism spectrum. The *summed* repressor plus DNA spectrum (curve c) of A has been subtracted from the *observed* repressor plus DNA spectrum (curve d).

taining column buffer. Fractions were collected from both the excluded volume (containing repressor) and the included volume (containing the solvent added with the repressor), and counted for [^{14}C]IPTG. A peak (or trough) of IPTG (relative to the column buffer background) was obtained, depending on the amount of IPTG present in the initial repressor-containing sample.

Sulfhydryl Modification Reactions. Aqueous stock solutions of Nbs_2 and DTP were stored in the dark at 4 °C. For experiments performed with IPTG or DNA mixed with repressor, these ligands were added at least 15 min prior to reaction to ensure repressor–inducer or repressor–DNA binding equilibrium. The progress of the modification reaction was followed by monitoring the increase in absorbance at 412 nm (for Nbs_2) or 324 nm (for DTP). Data were collected in digital form from a Cary 14 spectrophotometer interfaced to a Varian 620-i computer. The results were corrected for sulfhydryl reagent and protein blanks, and concentrations of reacted cysteines were calculated using an extinction coefficient (ϵ_{412}) of 1.36×10^4 M^{-1} cm^{-1} for Nbs_2 (Ellman, 1959), and (ϵ_{324}) 1.98×10^4 M^{-1} cm^{-1} for DTP (Grassetti and Murray, 1967).

Results

Molecular Weight Determinations. We determined the molecular weight of *lac* repressor directly by a sedimentation equilibrium method. This was done because most molecular weight measurements on *lac* repressor have been made by rather indirect means; e.g., sedimentation velocity (Gilbert and Müller-Hill, 1966), gel permeation chromatography (Riggs and Bourgeois, 1968; Laiken et al., 1972), etc. In addition, it was necessary to establish the molecular weight distribution of *lac* repressor under the environmental conditions used in our experiments. The results indicate that the native repressor preparations used in these studies are quite homogeneous under the experimental ionic conditions (no detectable curvature in plots of log c vs. r^2), and show weight average molecular weights of 140 000 (±5000) g/mol (based on a value for the partial specific volume of 0.741 mL/g, computed from amino acid composition by the method of McMeekin and Marshall, 1952). These values are in good agreement with the repressor tetramer molecular weight of 148 800 g/mol calculated from amino acid sequence data, and with the results of earlier measurements.

DNA Conformation Change on Binding lac Repressor. As indicated above, *lac* repressor shows a marked affinity for non-operator DNA. The data of Figure 1A, measured under conditions of tight repressor binding (see Revzin and von Hippel, 1977), show that the magnitude of the positive band (260–290 nm) of the circular dichroism (CD) spectrum of calf thymus DNA is markedly enhanced over the calculated sum of the separate DNA and repressor spectra by repressor binding. Figure 1B shows the difference spectrum obtained by subtracting curve c (the calculated sum of the repressor plus DNA curves) from curve d (the actual repressor plus DNA spectrum). Similar CD spectral changes on *lac* repressor binding have been obtained using phage λ DNA or poly[d(A-T)] (spectra not shown; see also Maurizot et al., 1974; von Hippel et al., 1975).

The observed spectral changes could, in principle, be due either to a change in the conformation of the protein or of the nucleic acid component, or both. However, to attribute CD changes of this magnitude (in the 260–290 nm region) to an alteration in repressor conformation would require a conformational reorganization of virtually the entire protein molecule. Furthermore binding to non-operator DNA appears to have no major effect on repressor structure; e.g., the intrinsic protein fluorescence spectrum and the affinity of repressor for inducer are unaltered by this interaction (Laiken et al., 1972; von Hippel et al., 1975; and see below). Thus we proceed on the more plausible assumption that the spectral changes represent primarily an alteration in the conformation of the nucleic acid.

Some insight into the possible nature of this conformational change can be obtained by comparing the repressor-bound DNA spectrum with appropriately normalized spectra for free A and B form DNA (Figure 2). It is apparent that the repressor-distorted DNA spectrum is very similar to the A form spectrum, in that both show a marked enhancement of similar magnitude of the positive (long wavelength) band. However, A form DNA also shows a blue-shifted crossover point (Tunis-Schneider and Maestre, 1970) which is not seen in the repressor-perturbed spectrum. This similarity suggests that one structural consequence of repressor binding might be some base-tilting, leading to the observed positive lobe enhancement and nonconservative CD spectrum. We note (see below and Revzin and von Hippel, 1977; Wang et al., 1977) that binding of repressor to non-operator DNA is not cooperative; this suggests that only some, rather than all, of the bases covered

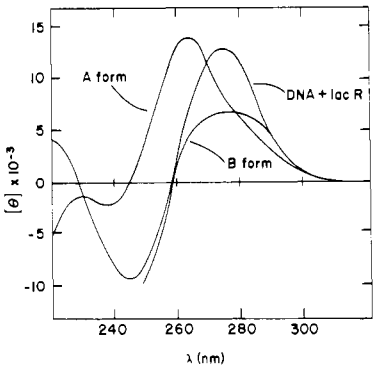

FIGURE 2: Comparison of circular dichroism spectra of A form and B form DNA with that due to DNA complexed with repressor. The data for the A form DNA spectrum are from Tunis-Schneider and Maestre (1970); the B form spectrum is that of calf thymus DNA.

by repressor may be tilted as a consequence of the binding (see discussion of the induction of protein binding cooperativity by DNA lattice distortion in von Hippel et al., 1977).

Another possible interpretation of the conformational change in DNA brought about by repressor binding may be based on the theoretical treatment of Cech et al. (1976). These workers suggest that the 275-nm positive CD band can be intensified by twisting the double helix (e.g., into a super-helical structure) in a way which brings neighboring base pairs into mutual orientations favoring increased exciton interaction.

The CD changes observed are clearly opposite in sign and magnitude to those induced by binding DNA melting proteins to single-stranded DNA (e.g., T4 gene 32-protein binding; Jensen et al., 1976); here the spectral changes have been interpreted in terms of unstacking and further separating neighboring bases, thus decreasing base-base interaction. Furthermore, though binding of *lac* repressor to non-operator DNA *stabilizes* the DNA against melting (Wang et al., 1977), the CD changes induced by this binding are also opposite in sign to those seen on the interaction of DNA with double-helix stabilizing histones in chromatin (e.g., see Sahasrabuddhe and Van Holde, 1974).

We therefore tentatively interpret the observed changes in the CD pattern of DNA resulting from repressor binding in terms of a partial base-tilting (toward the A form?) and/or a super-helical twisting which improves exciton coupling between vicinal bases. Additional studies will be required to further differentiate and specify these possibilities, as well as to determine whether the replacement of the solvent environment with protein *in itself* affects the CD spectrum of the DNA. In the next section we exploit this apparent conformational change to determine the site size for repressor binding to non-operator DNA.

Site Size for Repressor Bound to Non-Operator DNA. Circular Dichroism Titrations. By monitoring the change in molar ellipticity of repressor solutions (at constant repressor concentration) as a function of added DNA concentration under tight-binding conditions, we can determine the site size for *lac* repressor binding to non-operator DNA. Site size (n; in units of base pairs per repressor tetramer) is defined as the number of DNA base pairs covered (and thus not available for interaction with other repressor molecules) per repressor bound.

Figures 3A and 3B show the results of such CD titrations (at 270 nm) of repressor with wild-type (non-operator-containing) λ phage DNA and poly[d(A-T)], respectively. The titrations were conducted in a low salt buffer to ensure stoichiometric binding of the repressor to the added DNA (Revzin

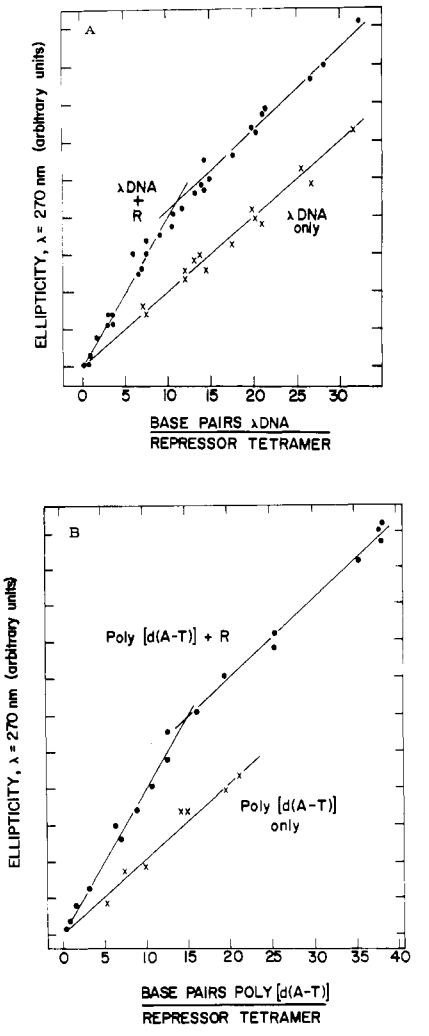

FIGURE 3: Titrations of non-operator DNAs with repressor, monitored by circular dichroism (see text). (A) Repressor plus λ DNA. (B) Repressor plus poly[d(A-T)]. Buffer: 0.02 M Tris, 10^{-4} M Na_2EDTA, pH 7.5; temperature = 27 °C. All data normalized to the same repressor concentration.

and von Hippel, 1977). As the first aliquots of DNA are added to the solution in such experiments [low DNA base pair/repressor ratios (r)], virtually all the DNA is covered with repressor; thus the slope of the line at low values of r reflects the enhanced CD spectrum due to the binding of repressor to DNA. At high values of r all the repressor is already bound to DNA, and the slope of the line (corresponding to the change in ellipticity upon further DNA addition) is identical with that of the lower line reflecting the ellipticity of DNA alone. The sharp break in the curve, where the slope changes from that characteristic of the complex to that due to DNA alone, provides a measure of site size for the non-specific binding of repressor to DNA. This value, corrected for incomplete binding due to overlap of potential binding sites,[2] is found to be ~12

[2] Under the conditions of this experiment (repressor in excess throughout the titration below the break-point), complete saturation of the DNA lattice is not achieved because of the unfavorable entropy involved in covering a lattice of potential overlapping binding sites with a noncooperatively binding ligand (McGhee and von Hippel, 1974). This leads to an overestimate of site size. However, under the ionic conditions of the experiment, repressor is bound very tightly, and we calculate that the lattice is at least 90% saturated. Thus the observed site size (13–14 base pairs) is corrected to a value of ~12 base pairs.

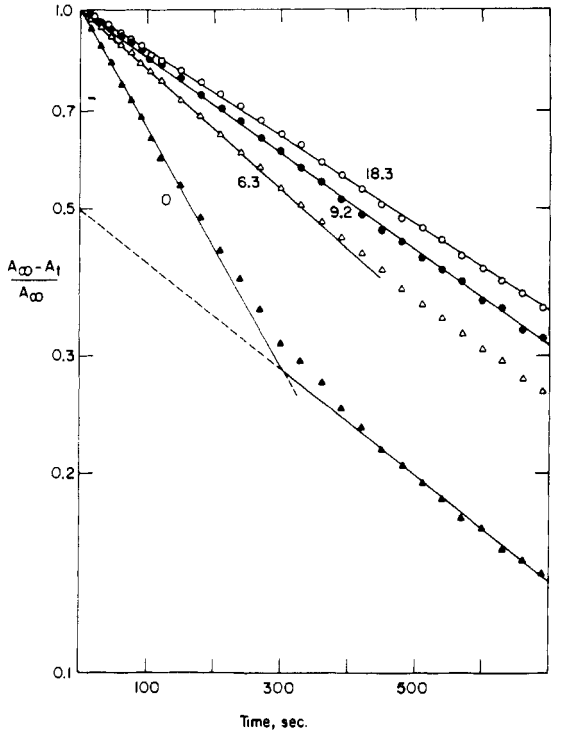

FIGURE 4: Reaction of repressor cysteines with 4,4′-dithiodipyridine (DTP). The increase in absorbance at 324 nm (corrected for sulfhydryl reagent and protein blanks) is plotted assuming pseudo-first-order reaction kinetics. The repressor (0.6 μM tetramer) was dialyzed extensively vs. 0.01 M K_2HPO_4, pH 7.6, and aliquots of a concentrated calf thymus DNA stock solution were added to give the indicated DNA/repressor ratios (base pairs/tetramer).

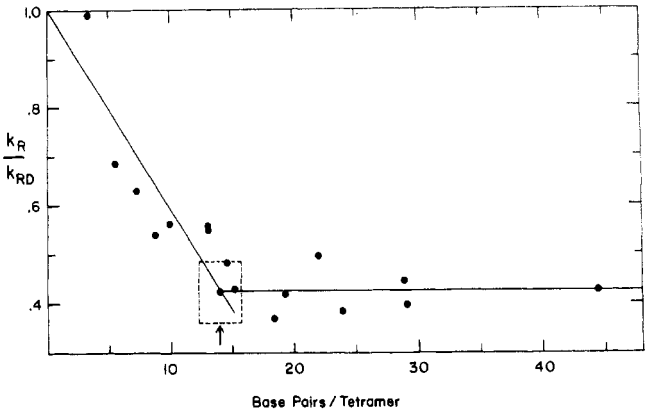

FIGURE 5: Titration of repressor cysteines as a function of DNA concentration. Rate constants were calculated from the initial slopes of curves such as those of Figure 4. These rates (k_{RD}) were normalized to k_R, the rate observed when no DNA was present in the reaction mixture. The unbroken lines were fit to the data using the method of least squares. The dashed rectangle defines the standard deviation of the intercept of these lines, which determines the site size.

base-pairs per repressor tetramer for both λ DNA and poly[d(A-T)].[3]

Binding Measurements by Sedimentation Velocity. The binding equilibrium of repressor with non-operator DNA has been analyzed by a sedimentation technique. A value of n consistent with a site size of ~12 base pairs can be obtained from these data (Revzin and von Hippel, 1977).

Sulfhydryl Titrations. The *lac* repressor contains three cysteine residues per subunit (Beyreuther et al., 1973). We have found that in native repressor two of these cysteine residues react at a reasonable rate with the sulfhydryl reagents 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) and 4,4′-dithiodipyridine (DTP); the third reacts only after complete denaturation of the protein. Inducer binding to *lac* repressor alters neither the rates nor extents of these reactions; however, binding of repressor to non-operator DNA does markedly affect the rates at which these reagents interact with the protein.

In a solution in which virtually all the repressor molecules are bound (non-specifically) to DNA, the initial rate of reaction of repressor with DTP is decreased ~2.5-fold (Figure 4), while the rate with Nbs_2 decreases ~15-fold (data not shown). The *difference* in the observed decrease in initial reaction rate for these two probes may reflect electrostatic repulsion of the negatively charged Nbs_2 by the DNA; the decrease in rate observed with DTP is assumed to arise mostly from steric blockage in access of the probe to the repressor as a consequence of DNA binding. This interpretation is reinforced by the observation that an equivalent concentration of free mononucleotides does not affect the reaction rates of these probes with repressor under identical ionic conditions. Furthermore, at higher salt (0.5 M Na^+), where essentially none of the added DNA is bound to the repressor, no depression in the reaction rate constants is observed.

Figure 4 shows that the kinetics of the interaction of DTP with repressor in the absence of DNA appears to be biphasic; ~50% of the reactive sulfhydryls appear to react more readily than the remainder, suggesting that the two available cysteine residues may have different microenvironments. Figure 4 also shows, in terms of this model, that as DNA is added the initially more reactive -SH group of repressor is substantially blocked as a consequence of DNA binding, while the rate of reaction with the initially less reactive species of cysteine seems largely unaffected. Thus in the presence of excess DNA the kinetics appear monophasic, with a rate constant characteristic of the less reactive -SH group in the uncomplexed native repressor.

In Figure 5 these data are replotted as the ratio of the initial rate of DTP reaction with DNA-complexed repressor (k_{RD}) over the initial rate in the presence of repressor alone (k_R), against the DNA-protein ratio (in base pairs per repressor tetramer). Again a fairly sharp break is apparent in the titration curve corresponding (after correction[2]) to $n \simeq 12$ (± 2) base pairs.

An interesting alternative interpretation of the kinetics of Figure 4 is suggested by Figure 5. Here we note that the total rate of reaction of repressor sulfhydryl groups with DTP is decreased approximately twofold by DNA binding. On this basis we may suggest that the accessible -SH groups of *two* repressor subunits are blocked by DNA binding, while those of the other two subunits remain unperturbed. This is in keeping with similar inferences derived from other aspects of the repressor–non-operator DNA interaction (see Revzin and von Hippel, 1977). This effect is less clear cut with Nbs_2, because long-range electrostatic interactions may perturb the kinetics of the interaction of this charged reagent with all the reactive groups of the repressor tetramer.

Number of Inducers Bound per lac Repressor Tetramer. Repressor contains four chemically and genetically identical subunits, each carrying a potential inducer binding site (for details see Bourgeois and Pfahl, 1976). Though many of our repressor binding experiments showed, as expected, four in-

[3] On the basis of such CD titrations, we had incorrectly reported (von Hippel et al., 1975) the site size for *lac* repressor binding to non-operator DNA as ~25–30 base pairs. This error resulted from calculating protein concentrations using the old (erroneous) value of the repressor extinction coefficient.

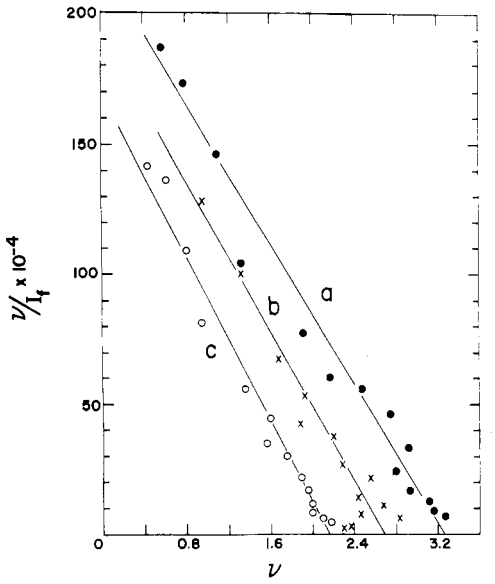

FIGURE 6: Equilibrium dialysis at 4 °C of several *lac* repressor (i^{SQ}) preparations. (a) Batch 23; (b) batch 24; (c) batch 23 after a second passage through phosphocellulose. Buffer: 0.2 M KCl, 0.01 M Tris, 10^{-4} M Na_2EDTA, pH 8.2 at 4 °C.

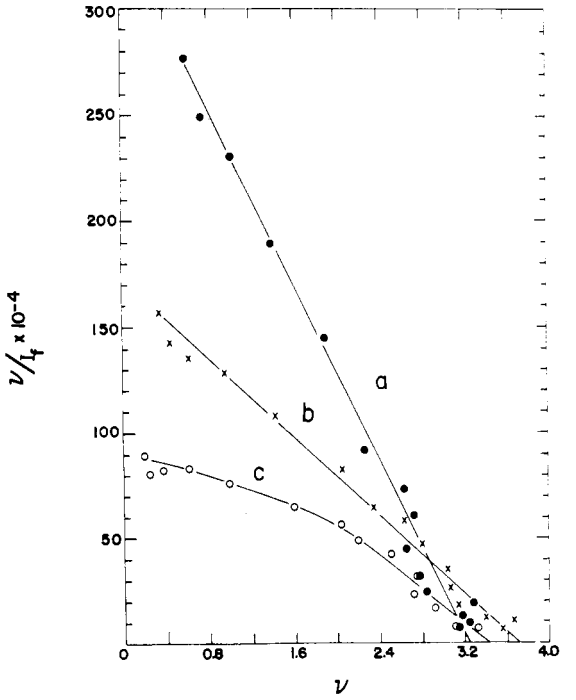

FIGURE 7: Equilibrium dialysis of *lac* repressor (i^{Q}, batch 30) at various temperatures. Each curve is the average of two or more experiments: (a) 4 °C; (b) 25 °C; (c) 37 °C. Buffer: 0.2 M KCl, 0.01 M (in Na^+) sodium phosphate, 10^{-4} M Na_2EDTA, pH 7.6 at each temperature.

ducer molecules bound per tetramer, a surprisingly large number seemed to extrapolate to saturation levels of three, or even two, inducer molecules bound per repressor. Since parallel developments in molecular enzymology (half-of-the-site reactivity, etc.; for a recent review, see Seydoux et al., 1974) have shown that such behavior may be of considerable regulatory and functional significance, we investigated this phenomenon further. After most of these studies were completed, a paper appeared by Ohshima et al. (1974) in which repressor molecules apparently containing less than four active inducer binding sites were also considered. The experiments presented below are complementary to those of Ohshima et al. (1974), and conclusions which can be reached considering both of these sets of experiments are described in the Discussion.

Equilibrium Dialysis. Experiments were performed as described in Materials and Methods. The data have been analyzed by scatchard binding plots, in which the ligand binding density, ν (in units of inducer bound per repressor tetramer), is plotted as abscissa against ν/I_f (I_f is the concentration of free inducer) as ordinate (Scatchard, 1949). If the repressor tetramer contains independent binding sites of equal affinity for inducer, the Scatchard plot will be a straight line with slope equal to $-K_{RI}$, where K_{RI} is the inducer-repressor association constant in M^{-1}. Regardless of possible interactions between binding sites, the horizontal axis intercept ($\nu/I_f = 0$) of the Scatchard plot must occur at $\nu = n_I$, the number of sites which are active (functional) for binding inducer under the experimental conditions used. Typical results obtained in equilibrium dialysis experiments using the "gratuitous" inducer, IPTG, are presented in Figures 6 and 7. The shapes of the curves will be discussed below; we first focus on the values of n_I extracted from these data.

Figure 6 shows that the n_I obtained depends on the particular repressor preparation used. Comparison of curves a and c in Figure 6 implies that repassage[4] of repressor over phosphocellulose may cause inactivation of some repressor sites with respect to inducer binding (although this observation yields no information on the cause or nature of the inactivation).

Values of n_I in the range 2.0 to 4.0 were obtained for a number of i^Q and i^{SQ} repressor preparations which were all more than 90% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We never obtained values of n_I greater than 4.0. The value of n_I obtained for a particular preparation was invariant with storage time (at −70 °C), at least over a period of several months. Meaningful correlation of the n_I values for each repressor preparation with the fraction of repressor active for operator binding in the filter assay was not possible due to the size of the experimental errors in the filter assay and to the uncertainties caused by the known differential rates of decay (with storage time) of the inducer- and operator-binding activities (Riggs et al., 1970).

For a given repressor preparation, we found that the value of n_I was little affected by pH in the range 7.4 to 8.3 (not all data shown). We note the interesting results of Ohshima et al. (1974), who reported n_I values of less than 4.0 at 4 °C, but found that n_I increased to 4.0 when the experiment was performed at 40 °C. These workers also indicated this change in n_I to be reversible, in that lowering the temperature caused n_I to revert to a value of less than 4.0. In our hands there appears, in general, to be a slight increase in n_I with temperature [compare curves a and b in Figure 7], although any such changes are not much larger than the experimental uncertainties. The same value of n_I (for a given repressor preparation) was obtained using buffers containing Mg^{2+} ions and buffers in which Mg^{2+} has been replaced with EDTA; thus we have not been able to affect the value of n_I significantly by either adding or removing Mg^{2+}. The value of n_I was also unaffected by the presence or absence of dithiothreitol.

This unexpected variability in n_I between repressor preparations led us to check these results using two other techniques.

[4] Gel electrophoresis of repressor used for curves a and c in Figure 6 showed that repassage over phosphocellulose resulted in only a very slight increase in the purity of the initially quite pure protein preparation.

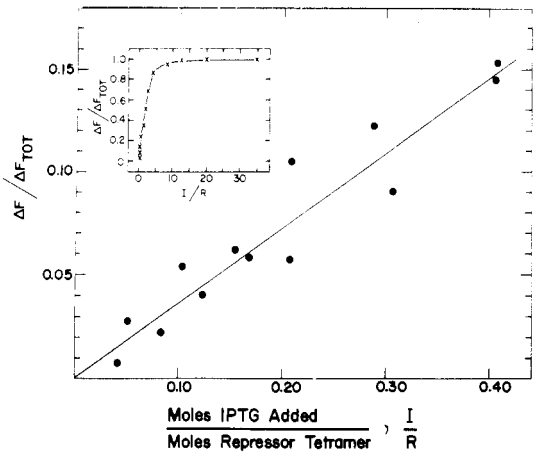

FIGURE 8: Fluorescence titration of *lac* repressor at 25 °C. Aliquots of IPTG, as indicated, were added to 2.6×10^{-5} M repressor. For this preparation (batch 24), $n_I = 2.7$ (compare Figure 6, curve b). The insert shows the complete titration curve. Buffer: 0.01 M Tris, 0.1 M NaCl, 10^{-4} M Na_2EDTA, pH 7.6 at 25 °C.

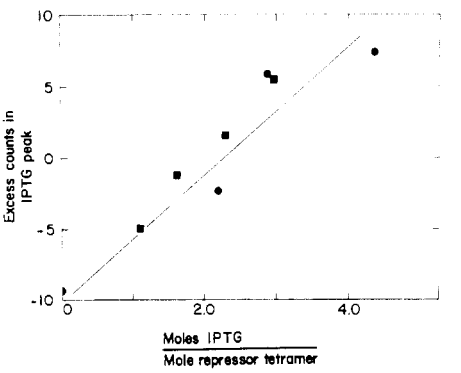

FIGURE 9: Binding of inducer to *lac* repressor, monitored by gel-permeation chromatography. Repressor (13.2 nmol in 0.7 mL of buffer) was applied to the column and eluted with 0.1 M NaCl, 0.01 M Tris, 10^{-4} M Na_2EDTA, pH 8.2 at 4 °C, containing 5.2×10^{-6} M [^{14}C]IPTG. The repressor had been preincubated with varying amounts (0 to 58 nmol) of [^{14}C]IPTG. Positive (negative) values on the ordinate correspond to excess (deficit) counts in the IPTG peak (trough) emerging at the included volume. Squares (■) and circles (●) refer to separate experiments on repressor preparation 24, for which $n_I = 2.3 \pm 0.4$.

Fluorescence Titrations. As an independent verification of the equilibrium dialysis results, the binding of IPTG to repressor was followed by a modification of the fluorescence assay developed by Laiken et al. (1972). Solutions containing repressor at concentrations of $\sim 3 \times 10^{-5}$ M (more than 10-fold greater than the repressor-inducer dissociation constant) were titrated with IPTG. At these protein concentrations binding of IPTG is expected to be essentially stoichiometric at low degrees of saturation. Thus a plot of $\Delta F/\Delta F_{tot}$ (the fractional saturation of the fluorescence change) vs. I/R (total moles of inducer added per mole of repressor tetramer) should be linear at small values of $\Delta F/\Delta F_{tot}$, with a slope of n_I^{-1} if binding is noncooperative.

Typical results of these fluorescence titrations are shown in Figure 8, for the same preparation for which equilibrium dialysis data are presented in Figure 6 (curve b). For this repressor preparation (and for others also checked, including both i^Q and i^{SQ}), we obtained virtually identical values of n_I with both experimental techniques.

Since repressor tends to aggregate with time at the relatively high repressor concentrations needed for these experiments, care had to be taken to avoid light-scattering artifacts. To this end a filter which excluded scattered exciting light of $\lambda < 310$ nm (or 350 nm) was installed on the exit side of the cell. Furthermore, we routinely performed the following control experiment. The fluorescence spectrum of an unaggregated repressor solution which had been brought to saturation with a single addition of inducer was compared with the spectrum of the somewhat aggregated sample which had been brought progressively to saturation by titration with inducer. The two spectra were identical. In addition, to check the possibility that repressor aggregation might lead to changes in inducer-binding stoichiometry, we performed some titrations using *lac* repressor bound to calf thymus DNA in 0.01 M KCl. When bound to DNA, repressor does not aggregate appreciably (Maurizot et al., 1974; Revzin and von Hippel, 1977; Wang et al., 1977). It has previously been shown that non-specific binding of repressor to DNA does not alter the repressor-IPTG association constant or the fluorescence properties of repressor (von Hippel et al., 1975). Identical values of n_I were obtained for free repressor, partially aggregated repressor, and repressor bound to non-operator DNA. Thus neither protein-protein aggregation nor non-specific DNA binding affects the repressor-inducer interaction stoichiometry.

We also examined the fluorescence quenching (at inducer saturation) of repressor preparations characterized by values of n_I ranging from 2.0 to 4.0. Results from ten different experiments indicated that the observed fluorescence quenching (at 360 nm) in the presence of saturating IPTG (i.e., $\Delta F_{tot}/F_{init}$) is 28 ± 2% for all the experiments, showing *no* correlation with n_I. This suggests that subunits which do not measurably bind IPTG in these preparations nevertheless undergo some of the conformational changes associated with inducer binding (Laiken et al., 1972) at very high inducer concentrations.

Gel Permeation Chromatography. Values of n_I for some repressor preparations were also established using the Hummel-Dreyer (1962) gel permeation chromatography technique. In these experiments, the repressor-bound IPTG emerges at the void volume as a peak of radioactivity above the background (free inducer) level. A [^{14}C]IPTG peak (or trough) corresponding to the amount of excess (or deficit) IPTG added with the protein emerges at the included volume. The amount of excess (or deficit) IPTG in this peak (or trough), plotted against the input ratio of molarity of IPTG to repressor tetramers, is shown in Figure 9 for a typical experiment. The concentrations of repressor and IPTG used were high enough to saturate all the inducer-binding sites. Under these conditions, n_I is the value of the abscissa at which the size of the included IPTG peak passes through zero (the estimated error in n_I determined by this technique is ±0.4). Again (compare with Figures 6 and 8), the values of n_I obtained in this way are virtually the same as those measured by equilibrium dialysis and fluorescence titration on the same preparations.

Other Parameters of the Inducer-Repressor Interaction. We now consider the *shapes* of the Scatchard plots measured by equilibrium dialysis. At 4 and 25 °C we see little, if any, curvature in these plots. (Some data at 4 °C seemed to show a slight curvature, but other experiments at this temperature yielded unambiguously straight lines; see Figures 6 and 7.) On the other hand, Scatchard plots measured at 37 °C can be definitely nonlinear (e.g., see Figure 7, data obtained at pH 7.6 and 37 °C). We obtained similar curves (data not shown) at pH 7.3 and 37 °C. Ohshima et al. (1974) have also reported curved plots at 40 °C, pH 8.0, but indicate they found no curvature at pH 7.0 and 40 °C. Such nonlinear Scatchard plots are diagnostic of positive cooperativity of binding of ligands (e.g., see McGhee and von Hippel, 1974), but, as pointed out by Ohshima et al. (1974), curvature of the magnitude observed

represents a change in the Hill coefficient of binding of ~0.1 or less.

The curvature in the higher temperature Scatchard plots is opposite to that which might result if the binding sites were heterogeneous in inducer affinity. The Scatchard plots obtained at lower temperatures are linear, also indicating that the binding sites are homogeneous. However, if the "inactive" subunits have an affinity for inducer which is less than 1/50 of that shown by the active subunits, then binding to the inactive sites would not be observed in these experiments.

The value of the repressor-inducer association constant (K_{RI}), derived from the Scatchard plots at 4 °C (pH 8.2) is 7.2 ± 0.5 × 10^5 M^{-1}. This value of K_{RI} varies little with the n_1 value of the repressor preparation used (compare slopes of the three lines in Figure 6). We find that the value of K_{RI} is essentially invariant with Mg^{2+} concentration (from zero to 10^{-3} M in 0.2 M KCl). This is of interest since Barkley et al. (1975) showed (at somewhat different ionic strengths) that Mg^{2+} has a considerable effect on the binding of inducer to repressor-operator complexes and on the affinity of repressor-inducer complexes for the operator region. We do find some dependence of K_{RI} on pH. Thus at pH 8.2 (4 °C), K_{RI} = 7.2 × 10^5 M^{-1}, while at pH 7.6 (4 °C), K_{RI} = 10.4 × 10^5 M^{-1}. A similar pH dependence was reported by Ohshima et al. (1974).

From our measurements of K_{RI} at 4 and 25 °C, pH 7.6 (Figure 7), we can make crude estimates of thermodynamic parameters of the repressor-inducer interaction. The standard free energy ($\Delta G°$) of association (per mole of active binding site) is −7.7 kcal/mol (at 25 °C); the standard enthalpy ($\Delta H°$) is −6.2 kcal/mol; and the standard entropy ($\Delta S°$) is +5 cal mol^{-1} deg^{-1} (at 25 °C). From these findings it appears that the inducer-binding process is largely enthalpy driven.

Discussion

Implications for lac Repressor-Operator Interactions. The results presented in this paper may provide some additional insight into the nature and geometry of the interaction of *lac* repressor with operator DNA. We find that the site size for the binding of *lac* repressor to non-operator DNA is 12 to 13 base pairs. This site size is in reasonable accord with other findings: Maurizot et al. (1974), using a light-scattering technique, have estimated that ~11 base pairs are covered in binding *lac* repressor to poly[d(A-T)]; Richmond and Steitz (1976), by cross-linking repressor to poly[d(A-U-HgX)] (where X represents a mercaptan), followed by nuclease digestion, have shown that repressor protects 13 to 16 base pair units of this double-helical polynucleotide against enzymic attack.

The site size of the operator region has been defined as the number of DNA base pairs protected against nuclease digestion by the presence of bound repressor. Gilbert and Maxam (1973) have demonstrated that the protected DNA fragment is 24 to 26 base pairs in length, or about *twice* the length of the non-operator site size. One might reconcile these different site sizes by arguing that *lac* repressor binds to non-operator sequences with a considerably different orientation or geometry than to the operator region. The following observations weigh against this possibility:

(1) *lac* repressor is negatively charged at physiological pH and shows a very similar ionic strength dependence of binding to operator and non-operator DNA (Riggs et al., 1970; Lin and Riggs, 1975b). This suggests that both types of binding involve the interaction of a "patch" of positively charged side chains on the repressor with the negatively charged DNA phosphate backbone. To account for the different site sizes then requires either that there be *two* such positive patches on the repressor, or else that the same patch can bind to DNA with two totally different geometries, which still show the same response to ionic strength changes.

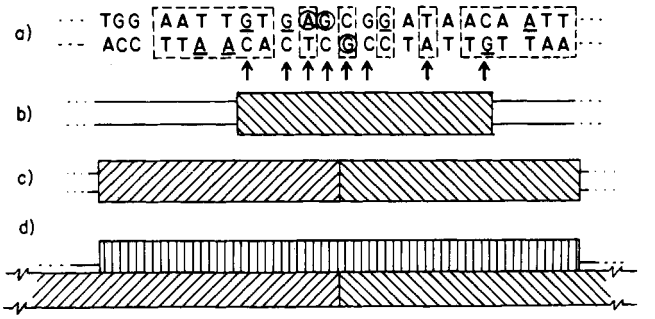

FIGURE 10: Schematic diagram of the (wild-type) *lac* operator region (those base pairs protected against nuclease digestion by bound repressor), indicating a possible binding site for one (b) or two (c) repressor tetramers per operator sequence. An alternate explanation for the observed non-specific site size is shown in d, where the repressor tetramers bind on both sides of the helix, each covering 26 *bases* (see text). Arrows (↑) indicate the sites of base pair changes in O^c mutations as determined by Gilbert et al. (1975). Boxed regions in a demonstrate loci of centrosymmetry. Underlined bases represent those protected from chemical methylation by bound repressor, and circled bases are those demonstrating repressor-enhanced methylation (Gilbert et al., 1976).

(2) Repressor from which the N-terminal peptides of the subunits have been cleaved, leaving only the inducer-binding tetrameric "core", does not bind operator (Platt et al., 1973) or non-operator DNA (von Hippel et al., 1975; Lin and Riggs, 1975a; Revzin and von Hippel, 1977). The N-terminal peptide region has a net positive charge at physiological pH (Platt et al., 1973) and has been shown genetically (Schlotmann et al., 1975) and biochemically (Jovin, Weber, and co-workers, in preparation) to be involved in both operator and non-operator DNA binding.

(3) Repressor binds preferentially to both double-stranded operator (Riggs et al., 1970) and double-stranded non-operator DNA (von Hippel et al., 1975; Wang et al., 1977).

(4) Inducer binds to repressor which itself is bound to either operator (Jobe and Bourgeois, 1972) or to non-operator DNA (Maurizot et al., 1974; von Hippel et al., 1975). Thus for either mode of repressor-DNA binding, sites on the protein are available for interaction with inducer.

(5) Repressor brings about some conformational change in DNA in binding to either operator (Wang et al., 1974) or to non-operator DNA (von Hippel et al., 1975; this paper).

It is obvious that all repressor functional groups involved in binding the operator are not engaged in non-operator DNA binding, since the latter binding is much weaker (Lin and Riggs, 1972), and inducer binding does not change the affinity of repressor for non-operator sequences (Lin and Riggs, 1972; von Hippel et al., 1975). Furthermore, Schlotmann et al. (1975) have shown that amino acids 1–52 of the N-terminal sequence of the repressor subunit are essential for both operator and non-operator DNA binding, while residues at positions 53–58 are involved only in the specific interaction with operator. Nevertheless, the arguments given above strongly imply that generally the same parts of the protein are involved in binding repressor to both operator and to non-operator sequences.

Given the conclusion that repressor binds in an overall similar manner to operator and non-operator DNA, there are at least three ways to reconcile the apparent differences in site size. One possibility is illustrated in Figure 10b, in which it is suggested that a single repressor molecule, physically covering 13 base pairs, binds at the center of the protected operator sequence (shown in Figure 10a). Since nuclease digestion was

brief in the Gilbert and Maxam (1973) experiment, 6 to 7 base pairs on either side of the DNA region actually covered by the repressor may be (sterically?) protected from DNase digestion.

Some evidence in support of this possibility may be derived from the DNA sequences of the operator regions of some operator-constitutive (O^c) mutations (Gilbert et al., 1975). Results currently available show that the base pair changes responsible for the mutations lie in the center of the protected operator fragment. Since O^c mutations have actually been *demonstrated* to lower repressor affinity for operator, at least in the simplest view the base pairs involved should make direct contact with repressor. As Figure 10b shows, a single, centrally placed repressor covering only the middle 13 base pairs of the operator fragment does indeed cover all the O^c-mutated base pairs.

In addition, very recently Gilbert et al. (1976) have reported experiments in which they examined the effects of repressor binding to operator on the reactivity of the various operator base pairs with dimethyl sulfate. They found that bound repressor modifies the rates at which the various bases are methylated only for certain bases within the central *17* base pairs of the sequence shown in Figure 10a.

We may also note that the "centrosymmetric" sequences found in the operator fragment lie largely outside of the central 13 base pair region, and hence might have little significance if the model depicted in Figure 10b is correct. Evidence that these centrosymmetric regions are *not* involved in "looped-out" cloverleaf conformations during repressor binding has been presented by Wang et al. (1974).

In Figure 10c we schematize an alternative model which involves the binding of *two* repressor molecules to the protected operator fragment. This model requires no ad hoc assumptions of protection against nuclease digestion beyond the actual covered site. Furthermore, data in the literature are not inconsistent with this model.

It has generally been assumed that one repressor molecule binds per operator. Riggs et al. (1970) showed, using the filter assay, that the repressor–operator binding curve is linear down to a repressor–operator molar ratio of 0.025. They pointed out that this indicates that one repressor molecule *is sufficient* to retain one molecule of operator-containing DNA on the filter. However, these data do not necessarily imply a one-to-one stoichiometry for repressor–operator binding. Indeed, Riggs et al. (1970) based their "one repressor per operator" hypothesis in part on the observation that the association constant derived from binding curves in which the concentration of repressor had been varied was the same as that obtained in experiments in which the operator concentration was changed. However, it is clear that, if the operator contains two binding sites having *different* affinities for repressor, then at certain ratios of affinities the second site will not be detectable within the accuracy of the filter assay.

Another argument that there is one repressor binding site per operator is derived from data which indicate that there are four inducer binding sites per operator site (Bourgeois and Jobe, 1970). These data were obtained using a crude repressor preparation in order to minimize any inactivation of repressor during purification. While there is no evidence that any repressor molecules were inactive with respect to either operator or inducer binding in these experiments, there is also no control to show that all the molecules were, in fact, fully active. The difficulty in obtaining repressor which is fully active in operator binding (Riggs et al., 1970) and the observation that n_I for inducer binding may be less than 4 under certain conditions (Ohshima et al., 1974; this paper) throw at least some doubt on the validity of the assumptions used in interpreting the results of Bourgeois and Jobe (1970).

Thus there are no incontrovertible data available which are not consistent with the possibility that there may be two repressor binding sites per operator region. These sites may have different affinities for repressor, although the association constants must be sufficiently large to permit both sites to be covered by repressor (and hence protected from nuclease digestion) in the procedure of Gilbert and Maxam (1973), who worked at relatively high concentrations of repressor and operator. There is also precedent for more than one repressor binding (with different affinities) to operator sequences in the λ phage system (Maniatis et al., 1973; Ptashne et al., 1976).

A third model which is also compatible with our data is that a single repressor tetramer might bind nonspecifically to cover 26 base pairs, but that a second repressor tetramer could bind on the opposite "side" of the helix, such that transverse sections through the DNA would bisect *two* repressor tetramers (Figure 10d). This model is also consistent with repressor–DNA titration curves showing one repressor molecule bound per 13 base pairs. This model seems somewhat less likely bebecause the circular dichroism titrations of repressor are linear as a function of added DNA, and because all repressor molecules seem to bind to DNA noncooperatively and with virtually identical affinities to the DNA lattice (Revzin and von Hippel, 1977). Thus if a repressor molecule binds noncooperatively to one "side" of a DNA double helix, and induces a certain conformational distortion in the DNA, this model requires that a second repressor subsequently binds on the opposite side of the DNA with apparently identical affinity and produces an apparently identical increment of change in the CD pattern of the system.

On the other hand, simple geometric considerations may be viewed as favoring models such as that of Figure 10d. Thus, assuming *lac* repressor tetramer is spherical, one can estimate the molecular diameter as ~70 Å. A protein of this size could cover ~20 base pairs along the DNA double-helix, and would be quite compatible with models such as Figure 10d. To reduce the distance covered along the DNA to ~44 Å (13 base pairs; Figures 10b and 10c) would place more stringent limitations on compatible repressor shapes and binding geometries. Intermediate situations, involving longer repressor molecules binding "at an angle" to the DNA double-helix and thus covering only ~13 base pairs each, are certainly also possible.

Implications for lac Repressor–Inducer Interactions. The results reported here, and in the work of Ohshima et al. (1974), show that different *lac* repressor preparations bind different numbers of inducer molecules per repressor tetramer. In our experiments, n_I, the number of IPTG molecules bound per tetramer at saturation, ranges from ~2.0 up to ~4.0 for these pure and otherwise indistinguishable protein preparations. Under any particular set of conditions, all "active" sites bind IPTG with comparable affinity, and (at least at temperatures below 30 °C) there is no intersubunit cooperativity of inducer binding. We have not found a reproducible procedure for interconverting active and inactive subunits. In one case repassage over phosphocellulose decreased n_I; also, in our hands a marginal increase in n_I with temperature was sometimes observed. Ohshima et al. (1974) report pronounced increases in n_I with temperature and state that these increases are reversible. This last finding, in particular, implies that the n_I differences are not trivial (i.e., uninteresting) covalent modifications of repressor caused by subtle differences in handling of repressor during purification, but indeed suggests that repressor subunits which are "active" and "inactive" with respect to inducer binding represent reversibily interconvertible species

which may have considerable physiological significance.

It is clear that repressor must be capable of assuming at least two conformations in vivo. In one of these conformations (R_O) it binds tightly to operator; the other conformation (R_I) is that assumed when inducer is bound to the protein. Based on the ratio of rates of β-galactosidase synthesis in induced and repressed cells (Jacob and Monod, 1961), and on in vitro filter assays (Jobe and Bourgeois, 1972), it appears that repressor in the R_O conformation binds about 1000 times more tightly to the operator region than does R_I repressor. The principle of microscopic reversibility then requires that the affinity of R_O for inducer also be much less than that of R_I for inducer. This conclusion is in accord with the recent experimental demonstration by Barkley et al. (1975) that the affinity of inducer for operator-bound repressor is indeed decreased by factors of 100 to 1000 from that characteristic of repressor free in solution. Additional experimental evidence that repressor molecules can exist in labile conformational equilibrium between two states comes from stopped-flow fluorescence studies of the kinetics of inducer binding to repressor. Laiken et al. (1972) found that the observed slow bimolecular rate constant for inducer binding could most easily be explained by postulating a prebinding conformational equilibrium between two forms of repressor subunit, with only one form appreciably active for inducer binding. Wu et al. (1976), by temperature-jump techniques, have directly demonstrated the existence of these postulated forms.

This set of observations leads us to hypothesize that, in repressor preparations showing $n_I < 4$, those subunits which are inactive in inducer binding are somehow "frozen" into an intermediate form lying between the R_O and the R_I conformations. This "frozen" form shows the weak inducer binding expected for R_O, but exhibits fluorescence changes suggesting at least a partial conformational transition to R_I. There is some precedent for the existence of such frozen intermediate conformations; Hatfield and Burns (1970), in studying conformational equilibria in the enzyme threonine deaminase, also invoked "frozen" conformations to explain some of their observations. Also very recently Betz and Sadler (1976) have made similar suggestions in connection with their studies of tight-operator-binding mutant *lac* repressors.

Obviously it would be of great interest to find conditions for the reproducible (and reversible) locking and unlocking of these conformations, perhaps by exposing the repressor to operator DNA, or by a modified temperature-cycling procedure based on the results of Ohshima et al. (1974). It will also be important to establish unambiguously whether the active and inactive subunits are combined into hybrid tetramers, or are segregated into totally active ($n_I = 4$) and totally inactive ($n_I = 0$) tetrameric species. The recent studies of Sadler and Tecklenburg (1976) and Geisler and Weber (1976) on in vitro "hybrid" repressor formation may provide valuable protocols for studies along these lines.

Note Added in Proof

Recently Zingsheim et al. (1977) have shown by electron microscopy that native DNA molecules completely coated with nonspecifically bound *lac* repressor look like flattened ribbons ~200 Å in diameter and ~70 Å high. Since free repressor tetramers visualized by this technique appear as rounded shapes ~85 Å in diameter, these dimensions are compatible with a row of repressor tetramers bound to *two* sides of the DNA double helix. This result supports model d of Figure 10 (this paper), and suggests that the binding site size, measured here as 12–13 base pairs assuming repressor binds to only *one* side of the DNA lattice, should actually be interpreted as n = 24–26 base pairs with binding on *both sides* of the lattice. We are grateful to Drs. Geisler and Weber for informing us of these studies prior to publication.

Acknowledgments

Some of this work has been presented to the Graduate School of the University of Oregon by A.P.B. in partial fulfillment of the requirements for the Ph.D. degree. We are very grateful to Pamela O'Conner and Ying Kao-Huang for preparing and purifying most of the *lac* repressor used in these studies, to Rebecca Hicks and Kathleen Sullivan for technical assistance, and to various colleagues in our laboratory and the Institute of Molecular Biology (including particularly Drs. James McGhee and Frederick Dahlquist), as well as to Drs. Robert Baldwin and Thomas Jovin, for helpful discussions of this work.

References

Barkley, M. D., Riggs, A. D., Jobe, A., and Bourgeois, S. (1975), *Biochemistry 14,* 1700.

Beaven, G. H., and Holiday, E. R. (1952), *Adv. Protein Chem. 7,* 319.

Betz, J. L., and Sadler, J. R. (1976), *J. Mol. Biol. 105,* 293.

Beyreuther, K., Adler, K., Geisler, N., and Klemm, A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3576.

Bourgeois, S., and Jobe, A. (1970), in The Lactose Operon, Beckwith, J. R., and Zipser, D., Ed., Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory, p 325.

Bourgeois, S., and Pfahl, M. (1976), *Adv. Protein Chem. 30,* 1.

Butler, A. P., Revzin, A., and von Hippel, P. H. (1976), *Biophys. J. 16,* 90a.

Cech, C. L., Hug, W., and Tinoco, I., Jr. (1976), *Biopolymers 15,* 131.

Clement, R., and Duane, M. P. (1975), *Nucleic Acids Res. 2,* 303.

Edelhoch, H. (1967), *Biochemistry 6,* 1948.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70.

Geisler, N., and Weber, K. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3103.

Gilbert, W., Gralla, J., Majors, J., and Maxam, A. (1975), in Protein-Ligand Interactions, Sund, H., and Blauer, G., Ed, Berlin, Walter de Gruyter, p 193.

Gilbert, W., and Maxam, A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3581.

Gilbert, W., Maxam, A., and Mirzabekov, A. (1976), The Alfred Benzon Symposium, Vol. 9, Copenhagen, Munksgaard, p 139.

Gilbert, W., and Müller-Hill, B. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 1891.

Grassetti, D. R., and Murray, J. F. (1967), *Arch. Biochem. Biophys. 119,* 41.

Hatfield, G. W., and Burns, R. O. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 1027.

Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63,* 530.

Huston, J. S., Moo-Penn, W. F., Bechtel, K. C., and Jardetzky, O. (1974), *Biochem. Biophys. Res. Commun. 61,* 391.

Jacob, F., and Monod, J. (1961), *J. Mol. Biol. 3,* 318.

Jensen, D. E., Kelly, R. A., and von Hippel, P. H. (1976), *J. Biol. Chem. 251,* 7215.

Jobe, A., and Bourgeois, S. (1972), *J. Mol. Biol. 72,* 139.

Kao-Huang, Y., Revzin, A., Butler, A. P., O'Conner, P., Noble, D. W., and von Hippel, P. H. (1977), *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Laiken, S. L., Gross, C. A., and von Hippel, P. H. (1972), *J. Mol. Biol. 66,* 143.
Lin, S.-Y., and Riggs, A. D. (1972), *J. Mol. Biol. 72,* 671.
Lin, S.-Y., and Riggs, A. D. (1975a), *Biochem. Biophys. Res. Commun. 62,* 704.
Lin, S.-Y., and Riggs, A. D. (1975b), *Cell 4,* 107.
McGhee, J. D., and von Hippel, P. H. (1974), *J. Mol. Biol. 86,* 469.
McMeekin, T. L., and Marshall, K. (1952), *Science 116,* 142.
Maniatis, T., Ptashne, M., and Maurer, R. (1973), *Cold Spring Harbor Quant. Biol. 38,* 857.
Maurizot, J.-C., Charlier, M., and Hélène, C. (1974), *Biochem. Biophys. Res. Commun. 60,* 951.
Mihalyi, E. (1968), *J. Chem. Eng. Data 13,* 179.
Müller-Hill, B. (1971), *Angew. Chem., Int. Ed. Engl. 10,* 160.
Ohshima, Y., Mizokoshi, T., and Horiuchi, T. (1974), *J. Mol. Biol. 89,* 127.
Platt, T., Files, J. G., and Weber, K. (1973), *J. Biol. Chem. 248,* 110.
Ptashne, M., Backman, K., Humayun, M. Z., Jeffrey, A., Maurer, R., Meyer, B., and Sauer, R. T. (1976), *Science 194,* 156.
Record, T. M., Jr., Lohman, T. M., and de Haseth, P. (1976), *J. Mol. Biol. 107,* 145.
Revzin, A., and von Hippel, P. H. (1977), *Biochemistry 16* (following paper in this issue).
Revzin, A., Wang, A. C., Gross, C. A., and von Hippel, P. H. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1463.
Richmond, T. J., and Steitz, T. A. (1976), *J. Mol. Biol. 103,* 25.
Richter, P. H., and Eigen, M. (1974), *Biophys. Chem. 2,* 255.
Riggs, A. D., and Bourgeois, S. (1968), *J. Mol. Biol. 34,* 361.
Riggs, A. D., Lin, S., and Wells, R. D. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 761.
Riggs, A. D., Suzuki, H., and Bourgeois, S. (1970), *J. Mol. Biol. 48,* 67.
Sadler, J. R., and Tecklenburg, M. (1976), *Biochemistry 15,* 4353.
Sahasrabuddhe, C. G., and Van Holde, K. E. (1974), *J. Biol. Chem. 249,* 152.
Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.
Schlotmann, M., Beyreuther, K., Geisler, N., and Müller-Hill, B. (1975), *Biochem. Soc. Trans. 3,* 1123.
Seydoux, F., Malhotra, O. P., and Bernhard, S. A. (March, 1974), *Crit. Rev. Biochem.,* 227.
Tunis-Schneider, M. J. B., and Maestre, M. F. (1970), *J. Mol. Biol. 52,* 54.
von Hippel, P. H., Jensen, D. E., Kelly, R. C., and McGhee, J. D. (1977), in Nucleic Acid-Protein Recognition, Vogel, H., Ed., New York, N.Y., Academic Press, p 65.
von Hippel, P. H., and McGhee, J. D. (1972), *Annu. Rev. Biochem. 41,* 231.
von Hippel, P. H., Revzin, A., Gross, C. A., and Wang, A. C. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4808.
von Hippel, P. H., Revzin, A., Gross, C. A., and Wang, A. C. (1975), in Protein-Ligand Interactions, Sund, H., and Blauer, G., Ed., Berlin, Walter de Gruyter, p 270.
Wang, A. C., Revzin, A., Butler, A. P., and von Hippel, P. H. (1977), *Nucleic Acids Res.* (Vinograd Memorial Issue) *4,* 1579.
Wang, J. C., Barkley, M. D., and Bourgeois, S. (1974), *Nature (London) 251,* 247.
Wu, F. Y.-H., Bandyopadhyay, P., and Wu, C.-W. (1976), *J. Mol. Biol. 100,* 459.
Zingsheim, H. P., Geisler, N., Mayer, F., and Weber, K. (1977), *J. Mol. Biol.* (in press).