Fluorescence and Chemical Studies on the Interaction of *Escherichia coli* DNA-Binding Protein with Single-Stranded DNA†

Pradip K. Bandyopadhyay and Cheng-Wen Wu*

ABSTRACT: Nanosecond and steady-state fluorescence spectroscopy were used to probe the environment of the tryptophan residues of *Escherichia coli* DNA-binding protein. A spectral shift and a change in quantum yield of the protein upon binding to DNA or oligonucleotides indicate that the tryptophan residues are near or at the DNA binding site. The observation of two excited-state lifetimes of the protein indicates that there is heterogeneity in the microenvironments of these tryptophan residues. The "short-lifetime" tryptophan residues are more sensitive to the interaction with DNA than the "long-lifetime" residues. The results of solute-perturbation studies with iodide or acrylamide indicate that there are tryptophan residues near the surface of the protein which are heterogeneous in their accessibility to these quenchers and that they become less accessible after DNA binding. Also, lysine residues of the protein have been shown to be essential to DNA binding by chemical-modification studies. Tyrosine, arginine, and cysteine residues appear not to be involved in this binding process. From studies of the decay of fluorescence anisotropy of the binding protein in the presence and absence of DNA, it has been concluded that (a) the tetrameric binding protein does not dissociate into subunits upon binding to the oligonucleotide $d(pT)_{16}$ and (b) the binding protein–fd DNA complex possesses "local flexibility" and, therefore, cannot be described as a continuous, rigid rod.

DNA–protein interactions play a vital role in cellular activity. These interactions can be broadly classified as specific or nonspecific. In specific interactions, the protein binds to a particular DNA sequence, e.g., binding of a repressor to an operator sequence or binding of RNA polymerase to a promoter sequence. There are a variety of proteins which bind to DNA without any sequence specificity. One class of such nonspecific DNA-binding proteins is commonly known as "unwinding proteins" (Alberts and Frey, 1970). These proteins have very low affinity for double-stranded DNA but bind tightly and cooperatively to single-stranded DNA. As a consequence, an "unwinding protein" can lower the melting temperature of duplex DNA. Recently, Alberts and Sternglanz (1977) have suggested the new term "helix-destabilizing protein" (HD protein) to designate such unwinding proteins. These proteins have been isolated from a variety of sources (phage, bacterial, and mammalian) and perform essential functions in DNA replication and genetic recombination.

Sigal et al. (1972) have described a DNA "unwinding protein" isolated from *E. coli*. This protein has a molecular weight of 80 000 and consists of four identical subunits. Like other "unwinding proteins", it binds tightly and cooperatively to single-stranded DNA but not double-stranded DNA. The stoichiometry of binding determined by electron microscopy (Sigal et al., 1972; Ruyechan and Wetmur, 1975) and fluorescence quenching measurements is approximately 32 nucleotides per protein tetramer (Molineux et al., 1975). In contrast, circular-dichroism studies indicate a stoichiometry of 56 nucleotides per protein tetramer (Anderson and Coleman, 1975). The *E. coli* DNA-binding protein is necessary for the in vitro conversion of single-stranded DNA to the replicative form in the replication systems of phage M13 (Geider and Kornberg, 1974) and phage G-4 (Weiner et al., 1975). It has been shown that the DNA-binding protein keeps single-stranded DNA in an extended configuration and stimulates the activity of *E. coli* DNA polymerases II and III but not DNA polymerase I or T4 DNA polymerase (Sigal et al., 1972; Molineux and Gefter, 1974). In addition, this protein also protects single-stranded DNA from nuclease digestion. Although a possible function for this protein has been suggested (Molineux et al., 1974), no definite assignment of a physiological role can be made in the absence of mutants impairing its activity.

In order to elucidate the molecular mechanism involved in the interaction of the binding protein with DNA, we have employed the techniques of fluorescence spectroscopy and chemical modification to probe the nucleic acid interaction site of the DNA-binding protein. Both structural and dynamic information obtained from these investigations is reported here. Physical studies on this protein have also been performed by other investigators (Molineux et al., 1975; Ruyechan and Wetmur, 1975, 1976; Anderson and Coleman, 1975).

Experimental Procedures

Materials

E. coli B was obtained from Grain Processing Corp. Phenyl glyoxal monohydrate was from Aldrich Chemical Co. Potassium iodide, iodine, and acetic anhydride were obtained from Fisher Scientific Co., and $d(pT)_{16}$ was purchased from P-L Biochemicals, Inc. *N*-(Iodoacetylaminoethyl)-5-naphthylamine-1-sulfonate synthesized by the method of Hudson and Weber (1973) was obtained from Dr. F. Y.-H. Wu of this laboratory. Acrylamide (Ultra Pure) was purchased from Polysciences, Inc., and *N*-acetyltryptophanamide from United States Biochemical Corp.

Methods

Preparation of DNA. DNA from bacteriophage fd was isolated and purified according to Molineux et al. (1975). Concentrations of single-stranded DNA and oligonucleotides were determined spectrophotometrically using extinction

† From the Department of Biophysics, Division of Biological Sciences, Albert Einstein College of Medicine, Bronx, New York 10461. *Received April 20, 1978.* This investigation was supported by research grants from the National Institutes of Health (GM 19062) and the American Cancer Society (BC 94).

0006-2960/78/0417-4078$01.00/0 © 1978 American Chemical Society

coefficients reported by Molineux et al. (1975). Single-stranded DNA-cellulose was prepared according to the method of Alberts and Herrick (1971).

Preparation of E. coli DNA Unwinding Protein. E. coli DNA unwinding protein was isolated according to the method described by Ruyechan and Wetmur (1975). The protein obtained in the final step from a DEAE[1]-cellulose column was dialyzed against 0.2 M NaCl, 0.01 M Tris-HCl (pH 8), 0.001 M EDTA, and 5% glycerol and stored at 4 °C until use. The protein was 98% pure as judged by $NaDodSO_4$-acrylamide gel electrophoresis. The protein was quantitated spectrophotometrically using the molar absorbtivity of $3 \times 10^4\ M^{-1}\ cm^{-1}$ at 280 nm based on a molecular weight of 20 000 (Ruyechan and Wetmur, 1975).

Steady-State Fluorescence Measurements. Steady-state fluorescence measurements were performed on a Perkin-Elmer MPF-3 fluorescence spectrophotometer provided with attachments for obtaining corrected spectra. Temperature was maintained constant at 25 °C using a cell which was appropriately thermostated. Absorbance was kept below 0.05 at the exciting and emission wavelength to eliminate errors due to the inner-filter effect. During titration, solutions were added from concentrated samples, and correction for dilution was made when necessary.

Solute-Perturbation Studies. These studies involve the quenching of the tryptophan fluorescence by low-molecular-weight quenchers (Lehrer, 1975), which decrease the fluorescence intensity of the residues via physical contact with the excited indole ring. Hence, the ease with which a fluorophore is quenched depends upon its "exposure" to the quencher. A convenient way to express this type of collisional quenching processes is described by the well-known Stern-Volmer relationship (Stern and Volmer, 1919):

$$F_0/F = 1 + K_Q[Q] = \tau_0/\tau$$

where F_0 and F are the fluorescence intensities and τ_0 and τ are the excited-state lifetimes of the fluorophore in the absence and presence of quencher, [Q] is the concentration of quencher, and K_Q is the collisional quenching constant. This relationship assumes that the contribution from "static" quenching (i.e., quenching due to ground-state complex formation) is negligible and its application is valid only if the fluorescence is homogeneous (Birks, 1970). For proteins containing more than one tryptophan residues, with each residue fluorescing independently, the equation can be written as:

$$F/F_0 = \sum \frac{f_i}{1 + K_{Qi}[Q]}$$

where f_i is the fraction of the total fluorescence corresponding to the ith tryptophan residue and K_{Qi} is the respective collisional quenching constant. Although this relationship is rather complex, the initial slope of the plot of F_0/F vs. [Q] is approximately equal to $\Sigma f_i K_{Qi}$, the weighted average of the individual quenching constants which will be referred to as the "effective" quenching constant.

Nuclease-Protection Assay. Binding protein in 0.1 M Tris-HCl (pH 8) and 0.2 M NaCl was mixed with denatured T7 [^{3}H]DNA, and the reaction mixture was incubated at 25 °C for 10 min. Twenty micrograms of DNase I and 2 μg of snake venom phosphodiesterase were added to the reaction mixture, and then $MgCl_2$ was added to a final concentration of 10 mM. This was incubated at 37 °C for 15 min. Perchloric acid at 0 °C was added to a final concentration of 1 M to stop the reaction, and the solution was incubated on ice for 5 min. After centrifugation, acid-soluble counts were determined by liquid scintillation counting. In a typical experiment, 8 μM T7 [^{3}H]DNA and 0.4 μM binding protein (tetramer) were used.

Fluorescent Labeling of Binding Protein. Binding protein was labeled with 1,5-I-AENS, and the extent of modification was estimated according to the methods described by Wu et al. (1974). A 15- to 20-fold molar excess of the dye was added to a solution of binding protein (0.2–0.5 mg/mL) in 0.05 M Tris-HCl (pH 8.5) and 0.2 M NaCl, which was incubated at 25 °C for 20 min and then at 5 °C for 8 h. The free dye was removed by gel filtration through Sephadex G-25 followed by extensive dialysis.

The labeled protein was centrifuged in a 5–30% (v/v) glycerol gradient in 0.05 M Tris-HCl (pH 8) and 0.2 M NaCl in a SW 50-1 rotor at 45 000 rpm for 15 h. Fractions containing the labeled protein were used directly for fluorescent measurements.

Excited-State Lifetime and Time-Dependent Emission Anisotropy Measurements. Measurements of the excited-state lifetimes were made by the single-photon counting technique using an Ortec 9200 nanosecond fluorescence spectrometer. An air lamp, pulsing at 25 kHz, was used when excitation was in the visible region. For excitation in the UV region, an Optitron Model NR-1 lamp filled with nitrogen gas at 200 psi was used. The exciting light was filtered through a Corning 7-37 filter and polarized in a direction perpendicular to the direction of incidence. The emitted light was passed through a Kodak Wratten 65 filter set at 90° to the direction of incidence. For anisotropy measurements, the emission polarizer was set at a direction parallel or perpendicular to the direction of polarization of the incident beam to obtain the parallel, $F_y(t)$, or perpendicular, $F_x(t)$, components of the fluorescence. The time dependence of the total fluorescence was measured by setting the emission polarizer at 54.7° with respect to the direction of the excitation polarizer.

Time dependence of the fluorescence intensity was recorded on a multichannel analyzer interfaced to a PDP-11 digital computer. The recorded fluorescence curve $R(t)$ is, however, distorted by the finite duration of the exciting pulse and the response time of the detection system. The time course of $R(t)$ is related to the true rate of emission $F(t)$ by the convolution integral

$$R(t) = \int_0^t L(t)F(t - T)\mathrm{d}T$$

where $L(t)$ is the exciting light pulse recorded by the fluorometer. The decay of $F(t)$ can be represented as a sum of exponential terms, depending on the number of "different" chromophores present.

$$F(t) = \sum a_i e^{-t/\tau_i}$$

Deconvolution was performed by the method of moments (Yguerabide, 1972). The parameters for best fit of $F(t)$ to either a single or double exponential were those which gave the smallest sum of weighted residuals. The deviation function in the ith channel, D_i, is defined as

$$D_i = \frac{V_i - F_i}{\sqrt{F_i}}$$

where V_i is the calculated convoluted value and F_i is the ex-

[1] Abbreviations used are: 1,5-I-AENS or I-AENS, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonate; $(pT)_{16}$, the oligonucleotide of thymidylic acid containing 16 residues; AENS-binding protein, *E. coli* DNA-binding protein modified with I-AENS; pCMB, *p*-chloromercuribenzoic acid; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; NBS, *N*-bromosuccinimide.

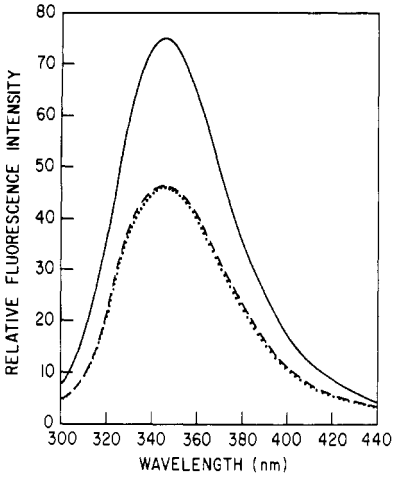

FIGURE 1: Fluorescence emission spectra of 5.6×10^{-7} M (tetramer) *E. coli* DNA-binding protein in 0.01 M Tris-HCl (pH 8) and 0.2 M NaCl: (—) protein excited at 280 nm; (- - -) protein excited at 296 nm; (• • •) normalized emission spectra of protein excited at 280 nm (see text).

perimental value of the counts in the ith channel.

In time-dependent emission anisotropy experiments, excitation with a very short pulse of y-polarized light produces an ensemble of excited molecules whose absorption dipoles are preferentially aligned along the y direction. The molecules then undergo Brownian motion, and their orientations become randomized. The rate of randomization depends on the size and shape of the molecule and also on its degree of flexibility during the excited-state lifetime of the fluorescence probe. The experimentally observed quantities are the time-dependence of fluorescence emission polarized along y direction, $F_y(t)$, and along a perpendicular direction x, $F_x(t)$. A very useful parameter derived from these data is the emission anisotropy, $A(t)$, which is defined as (Jablonski, 1962):

$$A(t) = \frac{F_y(t) - F_x(t)}{F_y(t) + 2F_x(t)}$$

For a rigid sphere, $A(t)$ decreases with time according to

$$A(t) = A_0 e^{-t/\phi}$$

where A_0 is the emission anisotropy at $t = 0$ and ϕ, the rotational correlation time, is proportional to the volume of the sphere. Thus, ϕ can be related to the molecular weight M, the specific volume ν, the hydration h of the molecule, the viscosity η, the absolute temperature T of the solution, the Avagadro number N, and the Boltzman constant k by:

$$\phi = \frac{M(\nu + h)\eta}{NkT}$$

For a rigid nonspherical particle, $A(t)$ decays according to a sum of exponential terms; the mean correlation time is longer than that of a rigid sphere of the same volume. In contrast, the rotational correlation time of a highly flexible particle is much less than that of a rigid sphere of the same volume.

Chemical Modifications of the Binding Protein. Reaction of the binding protein with *N*-bromosuccinimide was performed using the procedure described previously (Witkop, 1961; Koshland et al., 1963). The binding protein (3.3×10^{-7} M tetramer) was mixed with tenfold molar excess of *N*-bromosuccinimide in 2 mL of 0.2 M sodium acetate (pH 5). Incubation was carried out at room temperature until the fluorescence intensity of the protein decreased to less than 1% of the initial value. The reaction mixture was then dialyzed against 500 mL of 0.2 M NaCl and 0.1 M Tris-HCl (pH 8) for 3 h with three changes of the buffer, before using for nuclease protection studies.

To modify the binding protein with acetic anhydride (Fraenkel-Conrat, 1957), 0.2 mL of the protein (3.3×10^{-6} M tetramer) was mixed with an equal volume of saturated sodium acetate and chilled in ice. Five microliters of acetic anhydride was added to the mixture in ice. This was repeated once after 30 min. The incubation was continued for another 30 min, then the reaction mixture was dialyzed twice against 250 mL of 0.01 M Tris-HCl (pH 8), 0.2 M NaCl, and 5% glycerol for 5 h at 4 °C.

For modification with phenyl glyoxal (Takahashi, 1968), the reagent was dissolved in 0.1 M Tris-HCl (pH 8) and 0.2 M NaCl to a final concentration of 8–16 mg/mL. An aliquot of this solution was added to the binding protein (4×10^{-7} M tetramer) in the same buffer and incubated at 25 °C for a period of 10 min to 4 h. This reaction mixture was then used directly for nuclease protection studies. The amount of phenyl glyoxal added varied from 10- to 500-fold molar excess over the arginine residues in the binding protein.

Before iodination of the binding protein (Azari and Feeney, 1961), the protein (4×10^{-6} M tetramer) was dialyzed against 0.1 M sodium borate (pH 9.5) for 3 h at 4 °C. A 30-fold molar excess of KI_3 (from 0.05 M I_2 in 0.24 M KI) was added to the dialyzed protein solution which was chilled in ice. After 20 min, a drop of 1 M $NaHSO_3$ was added to stop the reaction. The iodinated binding protein was dialyzed extensively against 0.05 M Tris-HCl (pH 8), 0.2 M KCl to remove unreacted reagents.

Results and Discussion

Fluorescence Properties of the DNA-Binding Protein. E. coli DNA-binding protein contains three tryptophan and four tyrosine residues per monomer (Weiner et al., 1975). Figure 1 shows the fluorescence emission spectrum of the DNA-binding protein excited at 296 nm. At this wavelength, the absorption of light by a protein is almost entirely due to its tryptophan residues. The emission maximum is at 345 nm, in contrast to 355 nm for the model compound *N*-acetyltryptophanamide in the same buffer. When the binding protein was denatured in the presence of 8 M guanidine hydrochloride, the emission maximum shifted to 350 nm. These observations suggest that the tryptophan residues of native binding protein are located in an environment less polar than the aqueous solvent (Longworth, 1966; Teale, 1960).

For most proteins containing both tryptophan and tyrosine residues, tyrosine emission is usually negligible due to the high efficiency of energy transfer from tyrosine to tryptophan (Teale, 1960). Tyrosines far from tryptophan residues, however, would contribute independently to the protein emission spectrum. These "independent" tyrosine residues can be identified by a comparison of the protein emission spectra obtained by exciting at 296 and 280 nm (Eisinger, 1969). Both tryptophan and tyrosine residues will absorb at 280 nm. Since the emission at 370 nm is exclusively from tryptophan whether the excitation is at 296 or 280 nm, it is possible to normalize these two emission spectra. We have found that these normalized spectra for the DNA-binding protein are virtually identical (Figure 1). Thus, the excited-state energy of the tyrosine residues in the binding protein was either completely quenched or quantitatively transferred to tryptophan.

The quantum yield of the binding protein when excited at 296 nm was estimated to be 0.13 ± 0.01 with tryptophan as a standard of quantum yield 0.15 (Chen, 1967). The value of this quantum yield did not vary significantly over the excitation wavelength range 276–296 nm, again indicating that trypto-

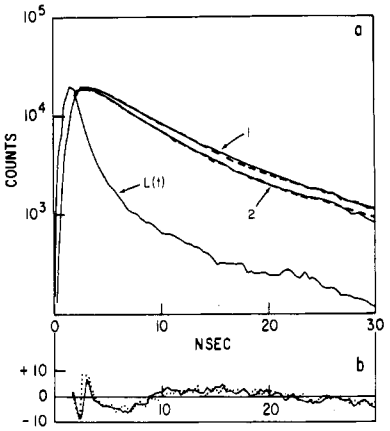

FIGURE 2: (a) Fluorescence emission kinetics of *E. coli* DNA binding protein excited at 296 nm. Binding protein, 1×10^{-6} M (tetramer), in 0.01 M Tris-HCl (pH 8) and 0.2 M NaCl. L(t) is the light pulse: curve 1, binding protein alone; curve 2, binding protein in the presence of 5.2×10^{-5} M (nucleotide) $d(pT)_{16}$. The dashed lines represent the convoluted fluorescence decay curves using the best fit parameters described in the text. (b) Deviation of the experimental curve from the fitted curve: (···) binding protein; (—) binding protein + $d(pT)_{16}$.

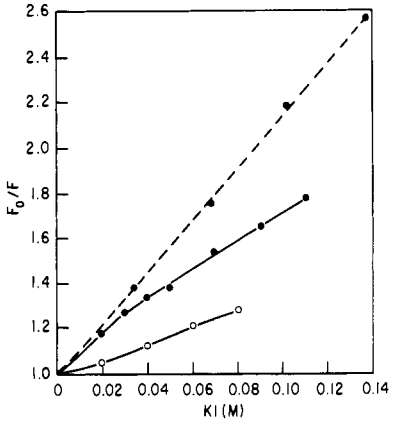

FIGURE 3: Quenching of *E. coli* DNA-binding protein with KI. Binding protein, 4.75×10^{-7} M (tetramer), in 0.01 M Tris-HCl (pH 8) and 0.2 M NaCl. Excitation wavelength was 296 nm and emission wavelength was 360 nm: (●-●) binding protein alone; (○-○) binding protein + 2×10^{-5} M fd DNA (in nucleotides); (● - - -●), *N*-acetyltryptophanamide.

phan residues are practically the only emitting species in the binding protein.

Excited-state lifetime measurements are more informative than quantum yield or other steady-state measurements in studying the microenvironments of fluorophores in macromolecules. For the tryptophan emission of *E. coli* DNA-binding protein, the nanosecond emission kinetics could be fitted by two excited-state lifetimes $\tau_1 = 4.1$ ns and $\tau_2 = 13.2$ ns, with relative amplitudes $\alpha_1 = 0.66$ and $\alpha_2 = 0.07$ (Figure 2). These results are indicative of heterogeneity of the microenvironments of the tryptophan residues in the binding protein.

A value of 13.2 ns for the lifetime of a tryptophan residue in a protein is rather long compared to typical values reported in the literature for other proteins.

Changes of the Protein Emission upon Binding to DNA. Quenching of the fluorescence of *E. coli* DNA-binding protein by single-stranded DNA or oligonucleotides was first reported by Molineux et al. (1975). We found that the addition of saturating concentrations of fd DNA decreased the quantum yield of the tryptophan emission of the binding protein from 0.13 to 0.025 without an apparent shift in the wavelength of the emission maximum. Similarly, in the presence of saturating $d(pT)_{16}$ the quantum yield of the binding protein decreased approximately 50% (to 0.07). The corresponding reductions in the short- and long-lifetime components of the protein were 15 and 4%, respectively ($\tau_1 = 3.5$ ns; $\tau_2 = 12.7$ ns; $\alpha_1 = 0.71$; $\alpha_2 = 0.07$). The differential changes in the two excited-state lifetimes suggest that the "short-lifetime" tryptophan residues are more sensitive to the interaction of the binding protein with DNA than the "long-lifetime" tryptophan residues.

It has been proposed by Brun et al. (1975) that tryptophan residues in a protein can interact with nucleic acids by stacking of the indole ring with purine or pyrimidine bases, resulting in complete quenching of tryptophan fluorescence. Using the tripeptide Lys-Trp-Lys as a model compound, these investigators observed that in the presence of saturating polynucleotide the quantum yield decreased by 50%, whereas the excited-state lifetime remained unchanged. This observation was interpreted as indicating the existence of only two types of peptide-DNA complexes: those which have the same fluorescence quantum yield and the same lifetime as the free peptide and those which are completely quenched and hence not detected in the fluorescence decay analysis. Our finding that $d(pT)_{16}$ induces smaller changes in the tryptophan lifetimes than in their quantum yield cannot be explained by the simple model of Brun et al. It seems likely that in the $d(pT)_{16}$-binding protein interaction some of the tryptophan residues are involved in "imperfect" stacking with the thymine bases, resulting in partial quenching and shortening of their excited-state lifetimes.

The oligonucleotides $d(pT)_{10}$, $d(pT)_{16}$, and fd DNA reduce the quantum yield of the binding protein by about 10, 50, and 80%, respectively, in the presence of 0.2 M sodium chloride. If tryptophan residues were located solely in the "binding domain" of the protein and nucleotides were "fed" into this domain, it is reasonable to expect that the oligonucleotides should, at saturation, yield the same extent of quenching, but this expectation is not borne out, implying, therefore, that some of the observed quenching with polynucleotides is brought about by changes in the protein conformation and that the final conformation assumed by the protein is a function of the length of the bound oligonucleotide chain.

Accessibility of Tryptophan Residues. As shown above, the tryptophan residues are involved in the interaction of the binding protein with DNA. We have attempted to probe the accessibility of the tryptophan residues by solute-perturbation studies. A number of ionic quenchers, such as I^- and NO_3^-, have been used extensively in studies with proteins (Lehrer, 1975). Figure 3 presents the Stern-Volmer plot for the quenching of the *E. coli* DNA-binding protein with an ionic quencher KI. The plot is indicative of there being heterogeneous tryptophan residues having widely different accessibilities to the quencher (Eftink and Ghiron, 1976). From the initial slope, a value of the effective K_Q was determined to be 9 M^{-1}. The effective K_Q is equal to $k_q(\text{eff})\tau_0^*$, where τ_0^* is the harmonic mean of the excited-state lifetime of the binding protein in the absence of quencher and $k_q(\text{eff})$, the effective rate constant for interaction of quencher with protein, can be taken as a crude estimate of the average exposure of the fluorescent residues in the protein. Using $\tau_0^* = 4.6$ ns, a value of the effective $k_q = 1.9 \times 10^9$ M^{-1} s^{-1} for the binding protein was calculated. Comparison of this value with $k_q = 4 \times 10^9$ M^{-1} s^{-1} for *N*-acetyltryptophanamide (obtained under similar conditions) indicates that the tryptophan residues are very accessible to iodide ions and hence are probably exposed on the surface of the protein. In the presence of fd DNA, these tryp-

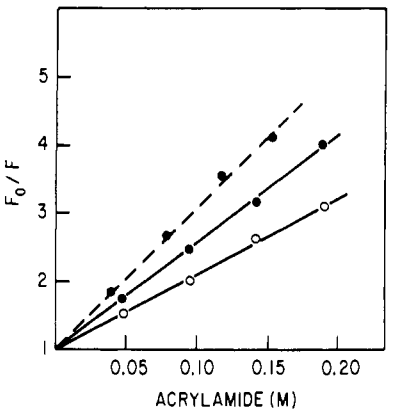

FIGURE 4: Quenching of *E. coli* DNA-binding protein with acrylamide. DNA-binding protein, 4.75×10^{-7} M (tetramer), in 0.01 M Tris-HCl (pH 8) and 0.2 M NaCl. Excitation was at 296 nm and emission at 360 nm: (●-●) binding protein alone; (○-○) binding protein + 1.9×10^{-5} fd DNA; (●- - -●) *N*-acetyltryptophanamide.

tophan residues become less accessible to iodide quenching. This is shown by the reduction of the effective k_q to 0.9×10^9 M^{-1} s^{-1} (Figure 3). Similar studies have been performed by Kelly and von Hippel (1976) on gene-32 protein, a DNA-binding protein of T4 phage. Tryptophan residues are also perturbed when this protein binds to single-stranded DNA. The Stern-Volmer quenching constant obtained for tryptophan residues in this protein is 2.2 M^{-1} compared to 9 M^{-1} for *E. coli* DNA-binding protein. The value obtained for denatured DNA-gene-32 protein is 0.9 M^{-1}, whereas for the *E. coli* binding protein it is ~3.3 M^{-1}. These data indicate that the tryptophan residues in *E. coli* DNA-binding protein are more exposed to the solvent than in the case of gene-32 protein, regardless of the presence of DNA.

Because both protein and DNA are polyelectrolytes, the solute-perturbation studies with ionic quenchers may lead to under- or overestimation of the accessibility of tryptophan residues due to electrostatic effects. To circumvent this problem, we have also performed quenching studies of the binding protein using acrylamide. Acrylamide is a polar, uncharged compound that has been shown to quench the fluorescence of indole derivatives predominantly by a collisional process (Eftink and Ghiron, 1976). The Stern-Volmer plot of the acrylamide quenching of the binding protein appears to be linear (Figure 4), indicating the existence of tryptophan residues differing slightly in accessibility (Eftink and Ghiron, 1976). The effective K_Q values in the absence and presence of fd DNA are 15.2 and 11.1 M^{-1}, corresponding to effective k_q values 3.3 and 2.9×10^9 M^{-1} s^{-1}, respectively. Under similar conditions, the values of K_Q and k_q for free *N*-acetyltryptophanamide are 21.0 and 7.5×10^9 M^{-1} s^{-1}, respectively. Again, these results are in accord with there being heterogeneous tryptophan residues near the surface of the protein, which become less accessible to solute after DNA binding. The difference in the quenching characteristics observed with KI and acrylamide may be attributed to the electrostatic effect. For example, the location of some tryptophan residues in "positive pockets" and others in "negative pockets" would result in differing accessibilities to the negatively charged iodide ion but not to the neutral acrylamide. Similarly, the fact that tryptophan residues are less accessible to iodide ions than to acrylamide in the presence of fd DNA is probably due to the repulsion of iodide ions by the negatively charged sugar-phosphate backbone of DNA.

Involvement of Other Amino Acid Residues in DNA Binding. To ascertain what amino acid residues are essential for DNA binding, we tested the DNA-binding ability of the binding protein after modification with various chemical reagents. The ability of the protein to bind DNA was assayed by its protection of single-stranded DNA against nucleolytic attack by snake venom phosphodiesterase (Ruyechan and Wetmur, 1976; Molineux and Gefter, 1975). In such chemical-modification studies, the assignment of which amino acid residue(s) are modified is based on the known specificity of the reagent; the possibility of another amino acid residue being involved cannot be ruled out, however. Table I summarizes the results of these chemical-modification studies.

TABLE I: Modification of *E. coli* DNA-Binding Protein with Various Reagents.

modifying reagent	most prob res modified	% protect. against nuclease act.[a]
NBS	Trp	0[b]
I_2/KI	Tyr	100
acetic anhydride	Lys	0
phenylglyoxal	Arg	100
1,5-AENS	Cys	100

[a] The 100% protection against nucleolytic attack on single-stranded DNA of a modified protein represents the extent of protection identical to that of unmodified protein under the same conditions. [b] The lack of nuclease protection may be due to cleavage of the protein by *N*-bromosuccinimide; see text.

When *E. coli* DNA-binding protein was treated with a tenfold molar excess of *N*-bromosuccinimide, the modified protein lost both its tryptophan fluorescence and DNA-binding ability completely. We have found, however, that at the concentration needed to modify tryptophan residues *N*-bromosuccinimide also cleaved the binding protein into smaller fragments. This was shown by $NaDodSO_4$-polyacrylamide gel electrophoresis.

When the binding protein was iodinated with a 30-fold molar excess of I_2/KI (Azari and Feeney, 1961) over the tyrosine residues in the protein and the excess reagent removed by extensive dialysis, we observed that the quantum yield of the iodinated binding protein diminished by 80% compared to uniodinated protein. It has been shown (Koshland et al., 1963) that under mild conditions the reaction of iodine with protein modifies primarily tyrosines, although under harsher conditions tryptophans and other amino acids can also be affected. That tryptophan residues were not significantly modified by iodination in our experiments was suggested by the observation that the quantum yield of the iodinated protein could be restored to almost the same level as that of unmodified protein upon denaturation with 6 M guanidine hydrochloride. Thus, the reduction in quantum yield of tryptophan emission observed upon iodination of the protein may be due to a direct interaction between tryptophan and the iodinated tyrosine residues. This is in agreement with the suggestion we made that in the binding protein tyrosine and tryptophan residues are in close proximity, so that the excited-state energy of the former is quantitatively transferred to the latter.

Arginine residues in proteins have been implicated in nucleic acid-protein interactions (Seeman et al., 1976). We have attempted to modify the arginine residues in the binding protein with phenylglyoxal (Takahashi, 1968), which reacts reversibly with guanidino groups. In the presence of a 500-fold molar excess of phenylglyoxal, we found that the ability of the binding protein to protect denatured DNA from nuclease digestion was not altered. Thus, it appears that the arginine residues are either not essential for DNA binding or are not available for modification under our experimental conditions.

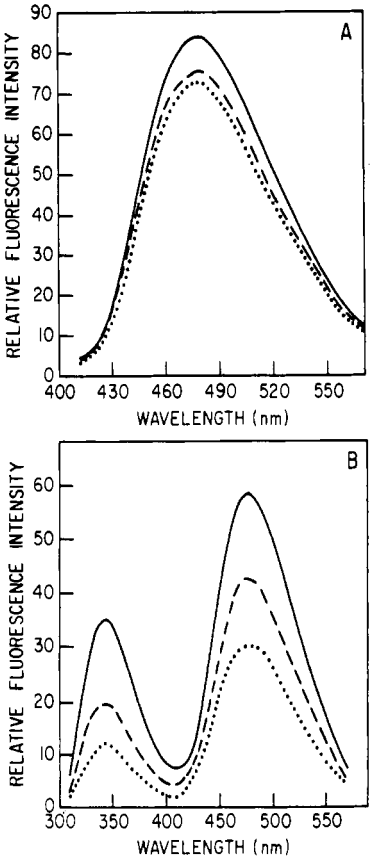

FIGURE 5: Emission spectra of AENS-binding protein in the presence and absence of $d(pT)_{16}$ and fd DNA excited at 350 (A) and at 296 nm (B). The buffer used was 0.01 M Tris-HCl (pH 8), 0.2 M NaCl, and 5% glycerol: (—) 6×10^{-7} M binding protein (tetramer); (- - -) 6×10^{-7} binding protein (tetramer) + 3.28×10^{-5} M $d(pT)_{16}$ (nucleotide); (· · ·) 6×10^{-7} M binding protein (tetramer) + 3×10^{-5} M fd DNA (nucleotide).

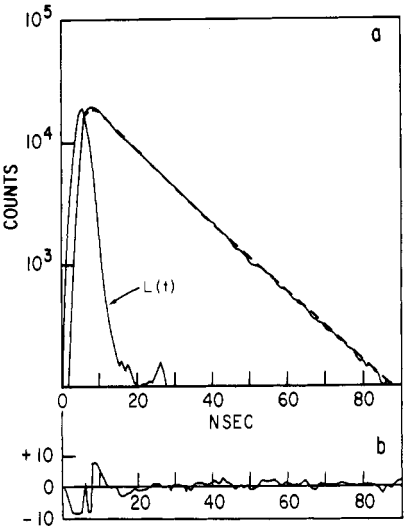

FIGURE 6: (a) Fluorescence emission kinetics of AENS-binding protein. Binding protein, 1×10^{-6} M (tetramer), in 0.01 M Tris-HCl (pH 8), 0.2 M NaCl, and 20% (v/v) glycerol at 15 °C. The dashed line represents the convoluted fluorescence decay curve using the best-fit parameters described in the text. (b) Deviation of the experimental curve from the fitted curve.

Lysine residues in the binding protein were modified by treatment with acetic anhydride (Fraenkel-Conrat, 1957). The acetylated binding protein completely lost DNA-binding activity. This was shown both by its inability to protect denatured DNA from nuclease digestion and the absence of any fluorescence quenching upon the addition of fd DNA (data not shown). Although the reaction conditions used here would allow selective modification of the ϵ-NH_2 group of the lysine residue, the OH group of the tyrosine residues may, to a lesser extent, also be susceptible to modification. However, the *O*-acetyltyrosine can be deacetylated by incubation in alkaline pH (11) at 37 °C for 4 h (Frankel-Conrat, 1959). The modified binding protein, which had been deacetylated this way, still failed to bind DNA after neutralization. In a parallel experiment, the DNA-binding activity of unmodified protein which was lost at pH 11 could be restored after neutralization. These studies indicate that the lysine residues in the protein are probably involved in DNA binding. The optimum pH range (7.5–8.5) for DNA binding by the protein may reflect the requirement for positively charged ϵ-amino groups in the DNA–protein interaction.

The binding protein contains one cysteine residue per monomer (Anderson and Coleman, 1975). When the protein was allowed to react with a 20-fold molar excess of 1,5-I-AENS, the number of AENS residues bound per protein monomer was estimated to be about 1.5. Analysis with radioactive pCMB revealed that the free SH group which was reactive toward pCMB in unmodified protein became unavailable to this reaction in AENS-labeled binding protein. Thus, it appears that I-AENS reacted with the single cysteine residue and probably also with some amino group in the protein. As indicated by the result shown in Table I, the AENS-binding protein is as effective as unmodified binding protein in protecting single-stranded DNA against nuclease digestion. This implies that the cysteine residue of the binding protein is not essential for DNA binding.

Fluorescence Studies with AENS-Binding Protein. Figure 5A,B shows the fluorescence emission spectra of AENS-binding protein in the presence and absence of oligo- and polynucleotides. A single emission maximum at 480 nm was obtained by excitation at 350 nm (Figure 5A). Addition of fd DNA or $d(pT)_{16}$ produced maximum quenching of 13 and 11%, respectively, without an apparent shift in the emission maximum. The fact that the quenching of the AENS emission was much smaller than that of the tryptophan emission (80 and 50% quenching) suggests that the binding of oligo- or polynucleotide did not result in a gross structural change of the binding protein. Any structural perturbation induced by DNA binding was probably localized in the region near the tryptophan residues. When the AENS-binding protein was excited at 296 nm, there were two emission maxima: one near 350 nm due to tryptophan emission and the other at 480 nm due to AENS emission (Figure 5B). The maximum fluorescence quenchings produced by the addition of fd DNA were 70% at 350 nm and 50% at 480 nm. The corresponding values for the addition of $d(pT)_{16}$ were 47% at 350 nm and 27% at 480 nm. A possible explanation for the larger quenching of AENS fluorescence by DNA when excitation was at 296 nm than when it was at 350 nm is that excitation energy transfer (Förster, 1965) due to a spectral overlap between the emission band of tryptophan and the absorption band of AENS could occur. Thus, the localized perturbation of tryptophan residues in the binding protein caused by DNA binding may also be reflected in the fluorescence change at 480 nm.

The fluorescence emission kinetics of AENS-binding protein was best fitted by a two-exponential decay curve with $\tau_1 = 11.0$ ns, $\tau_2 = 17.2$ ns and $\alpha_1 = 0.10$, $\alpha_2 = 0.09$ (Figure 6). The existence of two lifetimes may indicate that those AENS molecules which reacted with sulfhydryl groups and those which

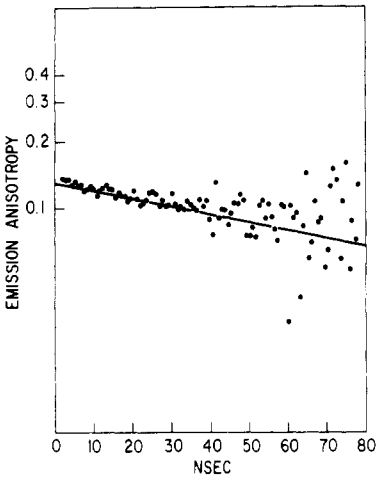

FIGURE 7: Time-dependent emission anisotropy of the AENS-binding protein. Binding protein, 1×10^{-6} M (tetramer), in 0.01 M Tris-HCl (pH 8), 0.2 M NaCl, and 20% (v/v) glycerol at 20 °C. Conditions for excitation and emission were as described in Figure 6. The straight line is the best fit of the data by a linear least-squares analysis.

reacted with amino groups are in different environments in the protein.

Rotational motions of the AENS-binding protein were studied by nanosecond fluorescence depolarization experiments. The time-dependent emission anisotropy plot [log $A(t)$ vs. t) for the AENS-binding protein is shown in Figure 7. To a first approximation, the plot is linear with a slope which gives a rotational correlation time of 108 ns. However, owing to the relatively short lifetime of the probe, a complete anisotropy decay curve covering a time range much longer than the rotation correlation time cannot be obtained. The data shown in Figure 7 exhibit only the early part of anisotropy decay, and hence ϕ = 108 ns calculated for the slope represents the harmonic mean of the rotational correlation times (Wahl, 1966). The radius of a rigid sphere having a rotational correlation time of 108 ns is 36 Å. This agrees very well with the Stokes radius of the binding protein being 38 Å (Weiner et al., 1975).

Molineux et al. (1975) observed that the $d(pT)_{16}$–binding protein complex sedimented with an apparent mass of 54 000 daltons. Thus, they concluded that the active binding species of the protein is either the dimeric or monomeric form. On the other hand, Ruyechan and Wetmur (1976) found that sedimentation of the binding protein in the presence of $d(pC\text{-}T)_{6\text{-}9}$ showed no gross change in protein structure. In order to resolve this discrepancy, we have performed time-dependent emission anisotropy measurements of the binding protein in the presence of $d(pT)_{16}$. No significant change (<10%) in the mean rotational correlation time was observed. Thus, our result is in accord with that of Ruyechan and Wetmur (1975), indicating that the protein species which binds to oligonucleotides of this length is tetrameric.

An important characteristic of the *E. coli* DNA-binding protein is its strongly cooperative binding to single-stranded DNA. It has been shown (Sigal et al., 1972) by electron microscopy that at a concentration of protein not sufficient to saturate all of the DNA some DNA molecules appear to be fully saturated with protein while others have little or no protein bound. The fully saturated DNA molecules exhibit an extended conformation. We have measured the time-dependent emission anisotropy of the saturated AENS-binding protein–fd DNA complex. At an ionic strength of 0.2, the average length of the binding protein–DNA complexes at 50% site coverage has been determined (Ruyechan and Wetmur, 1975) to be about 200 protein molecules. If these complexes maintained a rigid rod-like structure, they would exhibit a mean rotational correlation time at least on the order of μs.[2] Contrary to this expectation, we found that the mean rotational correlation time was 219 ns under these conditions. The low value of the rotational correlation time indicates the existence of local flexibility in the protein–DNA complex. It is very likely that the contiguously bound protein molecules on DNA are interspersed by small gaps of free DNA between protein stretches allowing some rotational freedom between them. Such small gaps of the order of few nucleotides cannot be detected by electron microscopy.

References

Alberts, B. M., and Frey, L. (1970), *Nature (London) 227,* 1313–1318.

Alberts, B. M., and Herrick, G. (1971), *Methods Enzymol. 21D,* 198–217.

Alberts, B. M., and Sternglanz, P. (1977), *Nature (London) 269,* 655–661.

Anderson, R. A., and Coleman, J. E. (1975), *Biochemistry 14,* 5485–5491.

Azari, P. R., and Feeney, R. E. (1961), *Arch. Biochem. Biophys. 92,* 44–52.

Birks, J. B. (1970), Photophysics of Aromatic Molecules, New York, N.Y., Wiley-Interscience.

Brun, F., Toulme, J., and Hélène, C. (1975), *Biochemistry 14,* 558–563.

Chen, R. F. (1967), *Anal. Lett. 1,* 35–42.

Eftink, M. R., and Ghiron, C. A. (1976), *Biochemistry 15,* 672–680.

Eisinger, J. (1969), *Biochemistry 9,* 3902–3908.

Förster, Th. (1965), in Modern Quantum Chemistry, Part III, Sinanoglu, O., Ed., New York, N.Y., Academic Press, p 93.

Fraenkel-Conrat, H. (1957), *Methods Enzymol. 4,* 247–269.

Geider, K., and Kornberg, A. (1974), *J. Biol. Chem. 249,* 3999–4005.

Hudson, E. N., and Weber, G. (1973), *Biochemistry 12,* 4154–4161.

Jablonski, A. (1962), in Luminescence of Organic and Inorganic Materials, Kallman, H. P., and Spruch, G. M., Ed., New York, N.Y., Wiley, p 110.

Kelly, R. C., and von Hippel, P. H. (1976), *J. Biol. Chem. 251,* 7229–7239.

Koshland, M. E., Englberger, F. M., Erwin, M. J., and Gaddone, S. M. (1963) *J. Biol. Chem. 238,* 1343–1348.

Lehrer, S. S. (1975), in Concepts in Biochemical Fluorescence, Chen, R., and Edelhoch, H., Ed., New York, N.Y., Marcel Dekker.

Longworth, J. W. (1966), *Biopolymers 4,* 1131–1148.

Molineux, I. J., and Gefter, M. L. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3858–3862.

Molineux, I. J., and Gefter, M. L. (1975), *J. Mol. Biol. 95,*

[2] The hydrodynamic radius of fd DNA, R_h, was determined by Newman et al. (1974) to be 31.6 ± 6 nm. Calculating from the relationship $\theta = kT/8\pi\eta R_h^3$, where k is the Boltzman constant, T is the absolute temperature, and η is the viscosity of the solution, the rotational diffusion coefficient θ for a rigid sphere of R_h = 31.6 nm is 0.65×10^4 s^{-1} at 20 °C in aqueous solution. Thus, the rotational correlation time of fd DNA would be $\phi_0 = 1/6\theta \simeq 25$ μs, assuming that it has a spherical shape. If fd DNA is a rigid rod, a shape that can be approximated by a prolate ellipsoid with a very long axial ratio, it would exhibit at least two rotational correlation times, the shortest of which is always longer than $0.95\phi_0$, the rotational correlation time of a rigid sphere having the same volume (Tao, 1969).

811-825.
Molineux, I. J., Pauli, A., and Gefter, M. L. (1975), *Nucleic Acids Res. 2,* 1821-1837.
Newman, J., Swinney, H. L., Berkowitz, S. A., and Day, L. A. (1974), *Biochemistry 13,* 4832-4838.
Ruyechan, W. T., and Wetmur, J. G. (1975), *Biochemistry 14,* 5529-5534.
Ruyechan, W. T., and Wetmur, J. G. (1976), *Biochemistry 15,* 5057-5064.
Seeman, N. C., Rosenberg, J. M., and Rich, A. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 804-808.
Sigal, N., Delius, H., Kornberg, T., Gefter, M. L., and Alberts, B. M. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3537-3541.
Stern, O., and Volmer, M. (1919), *Z. Phys. 20,* 183-188.
Takahashi, K. (1968), *J. Biol. Chem. 243,* 6171-6179.
Tao, T. (1969), *Biopolymers 8,* 609-632.
Teale, F. W. J. (1960), *Biochem. J. 76,* 381-388.
Wahl, Ph. (1966), *C. R. Hebd. Seances Acad. Sci. 263,* 1525-1528.
Weiner, J. H., Bertsch, L. L., and Kornberg, A. (1975), *J. Biol. Chem. 250,* 1972-1980.
Witkop, B. (1961), *Adv. Protein Chem. 16,* 221-321.
Wu, F. Y.-H., Nath, K., and Wu, C.-W. (1974), *Biochemistry 13,* 2567-2572.
Yguerabide, J. (1972), *Methods Enzymol. 26,* 498-578.

Isolation and Identification of 5,6-Epoxyretinoic Acid: A Biologically Active Metabolite of Retinoic Acid†

Anne M. McCormick, Joseph L. Napoli, Heinrich K. Schnoes, and Hector F. DeLuca*

ABSTRACT: A highly biologically active metabolite of retinoic acid (8_{III}) has been isolated in pure form from intestinal mucosa of vitamin A deficient rats given [^{3}H]retinoic acid. This metabolite has been positively identified as 5,6-epoxyretinoic acid based on the ultraviolet absorption spectrum and mass spectrum of its methylated derivative. This identification was confirmed by cochromatography of the methylated metabolite and synthetic methyl 5,6-epoxyretinoate on reverse-phase and straight-phase high-pressure liquid chromatography. The 5,6-epoxyretinoic acid is a true in vivo generated metabolite of retinoic acid and not an artifact of the isolation procedure. In addition, 5,8-oxyretinoic acid previously isolated in this laboratory from intestinal mucosa was probably generated from 5,6-epoxyretinoic acid by the acidic conditions used in the extraction and isolation of the 5,8-oxyretinoic acid.

Retinoic acid supports growth (Malathi et al., 1963; Krishnamurthy et al., 1963; Zile & DeLuca, 1968) and maintains epithelial differentiation (DeLuca et al., 1972), but it cannot support the visual (Dowling & Wald, 1960) or reproductive (Thompson et al., 1964) functions of vitamin A. Retinoic acid has been demonstrated to be a major metabolite in several tissues of rats given physiological amounts of retinol or retinyl esters (Kleiner-Bossaller & DeLuca, 1971; Dunagin et al., 1964; Deshmukh et al., 1965; Emerick et al., 1967; Crain et al., 1967). The sparing action of retinoic acid on depletion of liver retinol stores (Krishnamurthy et al., 1963; Krause et al., 1975; Nelson et al., 1972) and the similar metabolite profiles observed after administration of labeled retinol and retinoic acid (Roberts & DeLuca, 1967; Kleiner-Bossaller & DeLuca, 1971; Emerick et al., 1967; Ito et al., 1974) indicate that retinoic acid is a normal intermediate in vitamin A metabolism.

Retinoic acid is rapidly metabolized (Roberts & DeLuca, 1967; Fidge et al., 1968; Geison & Johnson, 1970; Kleiner-Bossaller & DeLuca, 1971) but to data no biologically active metabolites have been isolated from target tissues and positively identified. Retinoic acid is converted in vivo to polar metabolites designated as peaks 8, 9, and 10 which can be separated from retinoic acid by Sephadex LH-20 chromatography (Ito et al., 1974). With the development of high-pressure liquid chromatography (HPLC)[1] systems (McCormick et al., 1978a,b) for these metabolites, their isolation and identification became feasible. Thus a component of peak 8 from intestine of vitamin A deficient rats given retinoic acid was positively identified as 5,8-oxyretinoic acid (Napoli et al., 1978; A. McCormick, J. Napoli, H. Schnoes, & H. DeLuca, unpublished experiments). However, the use of an acidic extraction step in the isolation procedure introduced the possibility that the 5,8-oxyretinoic acid could have originated from 5,6-epoxyretinoic acid by acid catalysis (Morgan & Thompson, 1966; John et al., 1967).

This paper describes the isolation and chemical identification of 5,6-epoxyretinoic acid from intestines of vitamin A deficient rats given retinoic acid. Additionally, it will demonstrate that 5,8-oxyretinoic acid previously isolated from rat intestine is derived from 5,6-epoxyretinoic acid and that 5,6-epoxyretinoic acid is the major peak 8 metabolite of retinoic acid in rat intestine.

Experimental Procedure

Animals. Weanling male rats obtained from Holtzman Co. (Madison, Wis.) were maintained on a vitamin A deficient diet

† From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706. *Received April 18, 1978.* Supported by Program-Project Grant AM-14881 and Postdoctoral Training Grant DE-07031 from the National Institutes of Health and by the Harry Steenbock Research Fund.

[1] Abbreviations used: HPLC, high pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; BHT, butylated hydroxytoluene; ODS, octadecylsilane; THF, tetrahydrofuran; NH_4OAc, ammonium acetate.

0006-2960/78/0417-4085$01.00/0 © 1978 American Chemical Society