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## Accelerated Publications

# Fluorescence-Detected Circular Dichroism of Ethidium in Vivo and Bound to Deoxyribonucleic Acid in Vitro†

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ABSTRACT: Fluorescence-detected circular dichroism (FDCD) spectra are reported for ethidium in *Escherichia coli* cells and bound to *E. coli* DNA in vitro. FDCD bands are observed at 325 and 385 nm. These bands change amplitude as the ethidium to DNA ratio changes. Spectra are similar for in vivo and in vitro measurements. However, the bands at 325 and 385 nm disappear when ethidium binds to macromolecules without intercalating between base pairs. The results demonstrate that FDCD spectra can be measured in cell suspensions and indicate that ethidium binds to nucleic acids in *E. coli* cells by intercalation.

Physical–chemical studies have provided detailed information on the binding of drugs to nucleic acids in solution and in the solid state (Waring, 1981; Berman & Young, 1981; Krugh & Reinhardt, 1975; Jain et al., 1977; Tsai et al., 1977; Neidle et al., 1977; Wang et al., 1978; Quigley et al., 1980; Reinhardt & Krugh, 1978; Reuben et al., 1978; Pardi et al., 1983; Patel et al., 1981). These studies indicate drugs often bind to DNA by intercalation. The assumption is usually made that binding in vivo is similar. This is difficult to prove, especially since many additional receptors are present in a cell (Tritton & Yee, 1982). Fluorescence-detected circular dichroism (Turner et al., 1974; Turner, 1978) (FDCD)[1] is a spectroscopy that can be used both in vitro and in vivo and is sensitive to conformation (Turner et al., 1975; Lobenstine et al., 1981). This paper reports FDCD studies of ethidium bound to DNA in vitro and taken up by *Escherichia coli* cells in vivo. It provides the first measurements of FDCD on an in vivo system. The results indicate ethidium binds by intercalation in vivo as well as in vitro and demonstrate that FDCD can provide a spectroscopic bridge between in vitro and in vivo studies.

## Materials and Methods

Ethidium was purchased from Sigma and recrystallized from methanol. Stock concentrations were determined at 460 nm with $\epsilon = 5220\ M^{-1}\ cm^{-1}$. *E. coli* DNA was purchased from Sigma. The DNA was dissolved in 0.01 M sodium phosphate, 0.001 M NaEDTA, and 0.1 M NaCl, pH 7.0 (referred to as the phosphate buffer), homogenized, phenol extracted, and dialyzed against the phosphate buffer. Stock concentrations (in base pairs) were determined at 260 nm with $\epsilon = 13\,200\ M^{-1}\ cm^{-1}$. Coumarin 175 was obtained from Eastman Kodak. Stock concentrations were determined at 353 nm with $\epsilon = 1.61 \times 10^4\ M^{-1}\ cm^{-1}$.

The DM800 cells were incubated at 37 °C in LB broth (Miller, 1972) for 1.5 h (mid-log phase) and then incubated 15 min with either 0.2 or 0.02 mg/mL added ethidium. Cells were pelleted in a clinical centrifuge, rinsed twice with 0.01 M sodium citrate and 0.15 M NaCl, pH 7.4 (referred to as the citrate buffer), and taken up in citrate buffer to give an absorbance of 0.1 $cm^{-1}$ at 600 nm. FDCD spectra were taken immediately thereafter.

The cell membrane extract was prepared according to the method of Schnaitman (1981), with minor modifications described here. Incubated DM800 cells were pelleted and rinsed twice with citrate buffer; then they were resuspended in 10 mL of citrate buffer containing pancreatic ribonuclease and deoxyribonuclease (0.1 mg/mL each). The suspension was cooled to 4 °C and broken in a French Press operated at 10 000 lb/in.$^2$. After breakage, $MgCl_2$ was added to give a 1 mM total concentration, and the suspension was brought to room temperature (22 °C) for 30 min. Unbroken cells were removed by centrifugation at 5000*g* for 5 min (4 °C). The membrane was collected by centrifugation of the supernatant at 30000*g*

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[1] Abbreviations: FDCD, fluorescence-detected circular dichroism; CD, circular dichroism; bp, base pairs; EDTA, ethylenediaminetetraacetate.

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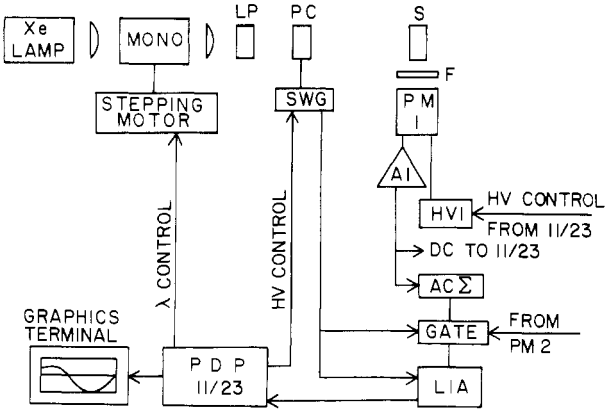


FIGURE 1: Block diagram of FDCD instrument. MONO is a Kratos GM252D double-grating monochromator; LP, a Glan-Thompson crystal polarizer; PC, the Pockels cell; SWG, the square wave generator; S, the sample cell; F, filters; PM 1, a Hamamatsu R375 photomultiplier; A1, a Pacific Instruments 3A14 preamplifier;HV 1, a Pacific Instruments 206-10L programmable dc high-voltage power supply; AC $\Sigma$, a circuit that sums the ac voltages from the two photomultipliers; LIA, a Princeton Applied Research 124A lock-in amplifier. Not shown is a second photomultiplier tube perpendicular to PM 1 and the excitation beam (Lobenstine et al., 1981). This tube has a separate preamplifier and high-voltage supply. The ac sum circuit adds the ac output of the two preamplifiers. The gate closes when the Pockels cell is switching between left and right circularly polarized light.

for 20 min (4 °C). The membrane was resuspended in citrate buffer and ethidium added to give total concentrations of 2 and 0.2 mg/mL.

CD and absorbance data were measured with a PDP-11/34 computer interfaced to a Jasco J-40 spectropolarimeter and a Perkin-Elmer 330 UV/vis spectrophotometer, respectively.

*FDCD.* FDCD measures the difference in fluorescence intensity excited by left and right circularly polarized light (Turner et al., 1974; Turner, 1978). Thus it combines the detection sensitivity and specificity of fluorescence with the conformational sensitivity of CD. Additional advantages are that it can be used on samples that are optically dense and/or highly scattering (White et al., 1975). The instrument used for these studies measures $2(F_L - F_R)/(F_L + F_R)$, where $F_L$ and $F_R$ are fluorescence intensities excited by left and right circularly polarized light, respectively. For samples with one fluorescent species, isotropic fluorescence, and no energy transfer, this is related to $\Delta\epsilon_F$ and $\epsilon_F$, the molar CD and absorptivity of the fluorophore by (Turner, 1978; Tinoco & Turner, 1976)

$$2\left(\frac{F_L - F_R}{F_L + F_R}\right) = \frac{\Delta\epsilon_F}{\epsilon_F} - 2R \qquad (1)$$

$$R = \frac{A_L(1 - 10^{-A_R}) - A_R(1 - 10^{-A_L})}{A_L(1 - 10^{-A_R}) + A_R(1 - 10^{-A_L})} \qquad (2)$$

Here $A_L$ and $A_R$ are sample absorbances for left and right circularly polarized light, respectively, and are determined from the absorbance and CD of the sample. For comparison with previous work [e.g., Lobenstine et al. (1981), Turner (1978), and Turner et al. (1974, 1975)], $\theta^\circ_F = -28.65(F_L - F_R)/(F_L + F_R)$.

$R$ corrects for differential absorption by the sample of left and right circularly polarized light. It can be determined separately by measuring $A_L$ and $A_R$ for the sample or by introducing a nonchiral, noninteracting, reporter fluorophore that fluoresces in a spectral region separate from the fluorophore of interest. In the latter case, $\Delta\epsilon_F/\epsilon_F = 0$ and the FDCD spectrum gives $-2R$ directly, when only the reporter fluorescence is detected.

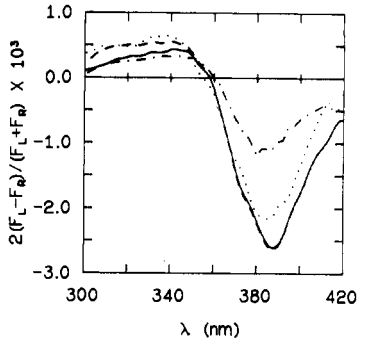


FIGURE 2: (—) FDCD spectrum of $1 \times 10^{-4}$ M bp *E. coli* DNA, $3 \times 10^{-5}$ M ethidium in 0.01 M sodium phosphate buffer, 0.001 M EDTA, and 0.1 M NaCl, pH 7. (---) Same as (—) but corrected for $R$ calculated from CD and absorbance to give $\Delta\epsilon_F/\epsilon_F$ (see eq 1). (-·-) FDCD spectrum of $1 \times 10^{-4}$ M bp *E. coli* DNA and $3 \times 10^{-5}$ M ethidium calculated from CD and absorbance spectra. (···) FDCD spectrum of DM800 *E. coli* cells grown at 37 °C in LB broth for 1.5 h and then incubated 15 min with 0.02 mg/mL added ethidium. Cells were pelleted in a clinical centrifuge, rinsed twice with 0.01 M sodium citrate and 0.15 M NaCl, pH 7.4, and taken up in the same buffer to give an absorbance of 0.1 $cm^{-1}$ at 600 nm.

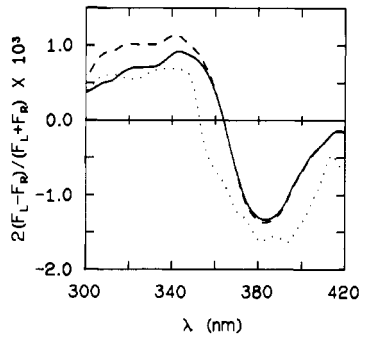


FIGURE 3: Same as Figure 2 but with (—) and (---) at $1 \times 10^{-4}$ M *E. coli* DNA and $5 \times 10^{-5}$ M ethidium. (···) DM800 *E. coli* incubated with 0.2 mg/mL ethidium.

The instrument used for these measurements is identical in principle with one described previously (Lobenstine et al., 1981), but has been redesigned to increase sensitivity. A schematic is shown in Figure 1. Light intensity has been increased a factor of 300 by incorporation of an ILC LX300W xenon lamp and a Kratos GM252D $f/3.6$ monochromator. Detection sensitivity has been improved by employing a Princeton Applied Research 124A lock-in amplifier and computer control by a PDP-11/23 for averaging of multiple scans. The overall sensitivity is 100-fold better than previous instruments.

For all ethidium spectra, Corning CS3-66 filters were used. For excitation wavelengths below 350 nm, the CS3-66 filters were preceded by Schott GG-10 filters. The cell path length was 1 cm. For coumarin 175 spectra, Corning CS5-59 filters preceded by Schott KV470 filters were used. This combination blocks ethidium fluorescence while transmitting coumarin 175 fluorescence. Cell path lengths of both 0.2 and 1 cm were used.

## RESULTS

FDCD spectra of $3 \times 10^{-5}$ and $5 \times 10^{-5}$ M ethidium with $1 \times 10^{-4}$ M *E. coli* DNA are shown in Figures 2 and 3, respectively. The largest band peak at 385 nm corresponds to an ethidium transition known to have a small $\epsilon$ but large $\Delta\epsilon/\epsilon$ (Hudson & Jacobs, 1975; Giacomoni & LeBret, 1973). The band centered at 325 nm arises from ethidium transitions centered at 335 and/or 300 nm. Above 300 nm, only ethidium

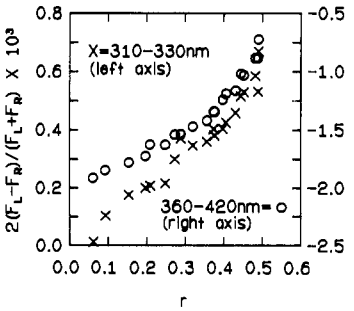


FIGURE 4: Average of $2(F_L - F_R)/(F_L + F_R)$ vs. $r$ for ethidium with $10^{-4}$ M bp *E. coli* DNA. Here $r$ = [bound ethidium]/[DNA, bp]. Averages from (×) 310–330 (left axis) and (○) 360–420 nm.

absorbs, and therefore the FDCD spectrum can be predicted from the CD and absorption spectra by using eq 1. The predicted spectrum is shown in Figure 2. This prediction is strictly valid only for depolarized fluorescence from a single species. However, the spectrum changes little when polarizers are placed in front of the photomultipliers, indicating polarization effects are small. As shown in Figure 2, the shape of the FDCD spectrum is accurately predicted, but the magnitude of the 385-nm peak is larger than predicted. This magnitude is dependent on base composition (data not shown) and the ratio of ethidium to nucleic acid (see Figure 4). Predicted and measured spectra are identical for $1 \times 10^{-5}$ M ethidium bound to $1 \times 10^{-4}$ M bp poly(dG-dC) (M. L. Lamos, G. T. Walker, T. R. Krugh, and D. H. Turner, unpublished results). Thus the difference between measured and predicted spectra may reflect the heterogeneity of binding sites in DNA.

Figures 2 and 3 show FDCD spectra of ethidium taken up by *E. coli* strain DM800, which shows enhanced uptake of the drug. For these experiments, *E. coli* was incubated in medium containing $5 \times 10^{-5}$ (Figure 2) or $5 \times 10^{-4}$ M (Figure 3) ethidium. Attempts were made to study cells grown in $5 \times 10^{-6}$ M ethidium, but fluorescence from these samples was only slightly larger than scattered light reaching the photomultipliers. Comparison of these spectra with those measured in vitro requires determination of $R$ in eq 1.

From 300 to 350 nm, $R$ was determined for both the in vitro and in vivo spectra by adding the nonchiral, negatively charged dye, coumarin 175, to the samples and measuring the FDCD spectrum with filters transmitting from 420 to 520 nm, where there is no fluorescence from ethidium. Visible CD spectra of coumarin 175 with DNA show no induced CD. DM800 cells incubated with coumarin and pelleted by centrifugation do not retain the dye. Thus it appears to be noninteracting with both free DNA and cells. For cell suspensions containing ethidium, coumarin 175 was added from $5 \times 10^{-6}$ to $1 \times 10^{-4}$ M, and the FDCD spectra were measured in 0.2- and 1-cm path length cells. For these spectra, $2(F_L - F_R)/(F_L + F_R) = 0$ from 300 to 350 nm.

$R$ for the in vitro samples was also determined by measuring $A_L$ and $A_R$ (eq 2). This determination was in excellent agreement with the coumarin 175 result from 300 to 350 nm. From 350 to 450 nm, $R$ was 0. This is expected since the CD of ethidium bound to DNA is smaller from 350 to 450 nm than from 300 to 350 nm (Aktipis & Kindelis, 1973). Figures 2 and 3 show spectra corrected for $2R$ to give $\Delta\epsilon_F/\epsilon_F$ (see eq 1).

The in vivo and in vitro spectra in Figures 2 and 3 are quite similar. Moreover, as ethidium concentration is lowered, the magnitude of the band at 385 nm increases both in vitro and in vivo. Ethidium is known to bind to nucleic acids in vitro by intercalation (Waring, 1981; Berman & Young, 1981; Krugh & Reinhardt, 1975; Jain et al., 1977; Tsai et al., 1977; Reinhardt & Krugh, 1978). Since CD is very sensitive to conformation, this suggests ethidium binds to nucleic acids in vivo by intercalation. Further support for this conclusion comes from FDCD spectra of ethidium bound to other macromolecules (data not shown). Spectra were measured for ethidium bound to liver glutamate dehydrogenase (Trivic & Leskovac, 1976), carboxymethylcellulose, poly(uridylic acid) (Kreishman et al., 1971), and a membrane extract of DM800 cells. Binding was confirmed by quenching of tryptophan fluorescence for dehydrogenase (Trivic & Leskovac, 1976) and changes in ethidium absorption with carboxymethylcellulose and poly(uridylic acid). No FDCD peaks were observed in the visible region for these samples. However, for ethidium bound to Phe-tRNA where intercalation is possible (Jones & Kearns, 1975; Jones et al., 1978), the FDCD spectrum is similar to those measured with DNA.

## Discussion

This paper reports the first FDCD measurements in vivo. FDCD has several advantages for such studies. It combines the high detection sensitivity and specificity of fluorescence with the conformational sensitivity of CD and can be used on scattering and optically dense samples. The similarity of FDCD spectra for ethidium bound to DNA in vitro and taken up by *E. coli* in vivo indicates ethidium binds in vivo by intercalation into nucleic acids. Recent results on the fluorescence lifetimes of ethidium in vitro and in vivo are consistent with this conclusion (Lambert & LePecq, 1984). The concentration of ethidium that could be monitored by FDCD was limited by interference from light scattering from the cell suspensions. This limitation can be eliminated by using pulsed excitation and time-gated detection. Thus it is possible to study very low concentrations of fluorophore. The method should be useful for studies of any process that can be monitored with a fluorescent probe and for diagnostic applications including cell sorting (Horan & Wheeless, 1977).

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**Registry No.** Ethidium, 3546-21-2.

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# Gramicidin K, a New Linear Channel-Forming Gramicidin from *Bacillus brevis*†

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ABSTRACT: A new gramicidin has been isolated from a commercial mixture of gramicidins A, B, and C. This new molecule, designated gramicidin K, contains formyl and ethanolamine blocking groups, has a molecular weight ~20% higher than gramicidin A, and is strongly retained on reversed-phase liquid chromatographic columns. Gramicidin K can be resolved into two components, one of which contains tyrosine. In lipid bilayer membranes, both components form channels of considerably longer lifetime and somewhat lower conductance than gramicidin A. Gramicidin K appears to be a lipopeptide that consists of a fatty acyl chain attached to the ethanolamine of gramicidin A.

The Dubos strain of *Bacillus brevis* (ATCC 8185) synthesizes the polypeptide antibiotics tyrocidine and linear gramicidin (Hotchkiss, 1944) by a nonribosomal polyenzyme template mechanism (Lipmann, 1973, 1980). The polyenzyme systems produce peptides of defined length, but the sequence fidelity of the process is somewhat lower than for ribosomal protein synthesis, and so some heterogeneity of the products is observed. The linear gramicidins, for example, are heterogeneous at positions 1 (valine or isoleucine) and 11 (tryptophan, phenylalanine, or tyrosine) in the sequence of 15 amino acids (Gregory & Craig, 1948; Ramachandran, 1963; Sarges & Witkop, 1965a–c). This heterogeneity is presumably due to a relaxed enzymatic specificity for the amino acid to be incorporated at each of these positions in the peptide (Lipmann, 1973). The major linear gramicidin produced by *B. brevis* is valine-gramicidin A, which has the amino acid sequence formyl-L-$Val_1$-$Gly_2$-L-$Ala_3$-D-$Leu_4$-L-$Ala_5$-D-$Val_6$-L-$Val_7$-D-$Val_8$-L-$Trp_9$-D-$Leu_{10}$-L-$Trp_{11}$-D-$Leu_{12}$-L-$Trp_{13}$-D-$Leu_{14}$-L-$Trp_{15}$-ethanolamine (Sarges & Witkop, 1965a). Gramicidin B has L-phenylalanine at position 11 and gramicidin C has L-tyrosine at position 11. These three peptides and their respective variants having L-isoleucine-1 in place of L-valine-1 can be separated by countercurrent distribution (Ramachandran, 1963) or by reversed-phase liquid chromatography (Koeppe & Weiss, 1981).

We now describe a new example of heterogeneity among naturally produced linear gramicidins: peptides that are larger and more hydrophobic than the previously described gramicidins. These new peptides as a group are designated gramicidin K. Two components of gramicidin K can be resolved: gramicidin K-C, which contains tyrosine, and gramicidin K-A, which does not. Like the familiar linear gramicidins A, B, and C, gramicidins K-C and K-A form cation channels having well-defined and distinctive conductances and lifetimes in lipid bilayer membranes. Gramicidin K was discovered in the course of chromatographic purification of gramicidins A, B, and C from commercially available mixtures, where we observed a compound that was strongly retained on reversed-

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