The interaction of diethylstilbestrol (DES) with calf thymus DNA was investigated at physiological pH with drug/DNA (phosphate) molar ratios (r) of 1:40, 1:20, 1:10, 1:4, 1:2, and 1. Fourier transform infrared and laser Raman difference spectroscopy were used to establish correlations between spectral changes and drug binding mode, sequence selectivity, DNA conformation, and structural properties of DES-DNA complexes in aqueous solution.

Spectroscopic results indicated that DES is a weak intercalator with affinity for A-T-rich regions. It is also a groove binder with a major interaction with the thymine O-2 atom. At low drug concentration (r = 1:40), the A-T-rich region is the main target of drug intercalation, while at a higher drug content (r > 1:5), external binding to the G-C bases also occurs with a partial helix destabilization. Evidence for this comes from the spectral alterations of the A-T vibrational frequencies at 1661 cm\(^{-1}\) (Raman) and 1663 and 1609 cm\(^{-1}\) (IR) and of the G-C vibrations at 1581 and 1491 cm\(^{-1}\) (Raman) and 1717 and 1492 cm\(^{-1}\) (IR). Drug intercalation leads to a major reduction of B-DNA structure in favor of A-DNA.

Diethylstilbestrol (DES)\(^1\) (Structure 1), a synthetic estrogen, is known to be a carcinogen in humans and in animals (1, 2). Radioactively labeled DES was found to bind to DNA in vivo (3-5) and in vitro (6), but the nature of complexation could not be clarified. The major difficulties in defining the nature of DES-DNA interaction were partially related to the instability of DES-DNA and DESQ-DNA complexes (DESQ is a metabolic intermediate product derived from DES oxidation) formed in vivo and in vitro (6, 7). Similarly, the possibility of intercalate formation of both DES and DESQ with DNA was excluded (7, 8). It has also been suggested that DES-DNA adduct formation may occur under oxidative stress (9, 10). However, a number of biological and biochemical effects of DES were noted that depended on metabolic activation of the stilbene and that are commonly associated with genotoxic activity (11). Since DES-induced carcinogenesis can be related to its complexation with DNA (2), the structural analysis of DES-DNA complexes has major biological importance, and thus, this investigation was undertaken. To our knowledge, there has also been no report on the interaction of DES with DNA using infrared or Raman spectroscopic techniques. Vibrational spectroscopy has been widely used as a major tool to characterize the nature of drug-DNA complexation and the effect of such interaction on the secondary structure of nucleic acids (13, 14). For example, in recent years, Raman and infrared spectroscopic techniques were applied to analyze the interactions between DNA and Adriamycin, 11-deoxyadriamycin (15), adriamycin, sain- topin (16, 17), anthracyclines (18), intopticine (13), doxorubicin (19), and platinum (20). Recently, we used FTIR and laser Raman spectroscopy to characterize the nature of DNA complexation with vitamin C (21) and aspirin\(^2\) in order to evaluate the effects of drug interaction on the DNA conformation.

In this work, we applied FTIR and laser Raman difference spectroscopy to analyze the structural properties of DES-DNA complexes formed at physiological pH with drug/DNA (phosphate) molar ratios (r) of 1:40 to 1. The FTIR spectra of DES-DNA complexes were compared with those of strong intercalating agents such as acridine orange, meseylene blue (MB), and ethidium bromide (EB), and the results are reported. Furthermore, the effects of DES-DNA interaction on biopolymer secondary structure and helix stability are evaluated.

**EXPERIMENTAL PROCEDURES**

Materials—Highly polymerized calf thymus DNA sodium salt (7% sodium content) was from Pharmacia Biotech Inc. and was used as supplied. DES was purchased from Sigma and was used without further purification. All other chemicals were reagent-grade.

Preparation of Stock Solutions—Sodium DNA was dissolved to 4% (w/v); 0.1 M NaCl in solution at 5 °C for 24 h with occasional stirring to ensure formation of a homogeneous solution. A solution of 1–50 mM DES was also prepared in water/ethanol solution (50:50, v/v). Mixtures of drug and DNA were prepared by adding DES solution dropwise to DNA solution with constant stirring to give the desired drug/DNA molar ratios of 1:40, 1:20, 1:10, 1:4, 1:2, and 1 at a final DNA concentration of 2% (w/w) or 0.05 M DNA (phosphate). Solution pH was kept near 7.5 to 6.5 with NaOH solution (0.1 M). The IR and Raman spectra were recorded 4 h after initial mixing of drug and DNA solutions.

Infrared spectra were recorded on a Bomem DA-3-0.02 Fourier transform infrared spectrometer with a nitrogen-cooled HgCdTe detector and a KBr beam splitter. Solution spectra were taken using AgBr windows with a resolution of 2 cm\(^{-1}\) and 100–500 scans. Water subtraction was carried out as reported (23). A good water subtraction was achieved as shown by a flat baseline around 2200 cm\(^{-1}\), where the water combination mode is located. The FTIR difference spectra (DNA solution + DES solution) – DNA solution) were produced using the band at 968 cm\(^{-1}\) as an internal standard. This band related to deoxyribose C-C stretching vibrations, exhibits no major alterations (intensity or shifting) on DES-DNA complexation, and is canceled upon spectral subtraction.

Laser Raman spectra were recorded on a Dilor Omars-S9 Raman spectrometer using a 514.4-nm line of an argon laser (Spectra-Physics Model 2020-03). The laser power was 300 milliwatts at the sample. The spectra were typically recorded at a 5 cm\(^{-1}\) slit width with a 2-s integration time at a 2 cm\(^{-1}\) frequency increment. The spectra were routinely background-corrected by subtracting an appropriate third.

\(^1\)The abbreviations used are: DES, diethylstilbestrol; DESQ, diethylstilbestrol-4'-quione; FTIR, Fourier transform infrared; r, drug/DNA (phosphate) molar ratio.

\(^2\)Neault, J.-F., Naoui, M., Manfait, M., and Tajmir-Riahi, H.-A., FEBS Lett., in press.
DE S
Structure 1.

degree polynomial function from the original curve. The spectra presented here are not smoothed. The difference spectra (DNA solution + DES solution) - DNA solution) were obtained according to our previous report (24) using the intense Raman line at 787 cm\(^{-1}\) as internal reference. This line, due to the coupling of cytosine and phosphodiester modes, exhibits no major spectral changes (intensity or shifting) on drug-DNA interaction and is canceled upon spectral subtraction.

The intensity ratios of several DNA in-plane vibrations (against the band at 968 cm\(^{-1}\)) related to the A-T and G-C base pairs were measured as a function of DES concentration with error of \(\pm 5\%\). The detailed infrared spectral manipulations and intensity ratio calculation are presented in our recent report (25).

RESULTS AND DISCUSSION

DES-DNA Complexes—At low DES concentration \((r = 1:40)\), a minor increase in the intensity (20%) of several infrared bands was observed at 1717 cm\(^{-1}\) (G and T), 1663 cm\(^{-1}\) (T, A, G, and C), 1609 cm\(^{-1}\) (A), and 1492 cm\(^{-1}\) (C and G) related to both A-T and G-C in-plane vibrational frequencies (23–30). These intensity variations were also associated with a minor shift of the band at 1717 cm\(^{-1}\) toward a lower frequency at 1715 cm\(^{-1}\) (Figs. 1 and 2). On the other hand, the Raman spectra of DES-DNA complexes formed at a low drug content \((r = 1:40)\) exhibited a major decrease (50%) in the intensity of the thymine line at 1661 cm\(^{-1}\) (30) with the shift of this band toward a higher frequency at 1669 cm\(^{-1}\) (Fig. 3). The presence of a negative derivative feature at 1669 cm\(^{-1}\) in the Raman difference spectrum of the DES-DNA complex is due to a major loss of intensity and shifting of the thymine line at 1661 cm\(^{-1}\) (Fig. 3, \(r = 1:40\)). Since the major spectral changes observed are for the thymine bands at 1663 cm\(^{-1}\) (IR) and 1661 cm\(^{-1}\) (Raman), drug interaction is mainly with the thymine O-2 atom with minor intercalation along the A-T-rich region. A similar increase in the intensity of several A-T Raman lines was observed upon metalloporphyrin intercalation with poly(dA-dT)\(_2\) biopolymer (33, 34).

Theoretical calculations on DNA fragments have suggested (31, 32) that the minor groove frequently carries a strong negative surface potential, and therefore, strong electrostatic interactions between the positive center (on the intercalating agent) and the negative charges found in the minor groove may facilitate the external binding mode. External binding to the A-T-rich region was also found in a series of DNA intercalators (34–36).

Evidence for no major drug interaction with the G-C region comes from only slight alteration of the guanine and cytosine lines at 1580 cm\(^{-1}\) (G), 1490 cm\(^{-1}\) (G), and 1257 cm\(^{-1}\) (C) in the Raman spectrum of the DES-DNA complex (Fig. 3, \(r = 1:40\)). However, it should be noted that drug intercalation with the A-T base pairs is weak since the overall spectral changes (intensity variations) of the A-T bands are not so large. The addition of more drug \((r > 1:40)\) leads to DES-DNA complexation via the A-T base pairs in a similar fashion with no major alteration of the helix structure (Fig. 2).

At \(r > 1:10\), a minor reduction of intensity (20%) was observed for the A-T infrared bands at 1663 and 1609 cm\(^{-1}\) upon drug interaction (Fig. 2). The reduction in the intensity of the A-T vibrations was associated with the shift of the PO\(_2\) antisymmetric vibration at 1222 cm\(^{-1}\) to 1232 cm\(^{-1}\) (Fig. 1). These infrared spectral changes were accompanied by the shifting of the Raman marker line (B-DNA) at 838 cm\(^{-1}\) (phosphodiester mode) toward a lower frequency at 832 cm\(^{-1}\) (Fig. 3). These spectral changes are due to a partial alteration of the A-T backbone B-structure toward A-DNA conformation upon drug interaction.

At high DES concentration \((r > 1:5)\), a major intensity increase (60%) was observed for the G-C bands at 1717 and 1492 cm\(^{-1}\) as well as for the A-T vibrations at 1663 and 1609 cm\(^{-1}\).

FIG. 1. FTIR spectra and difference spectra (DNA solution + DES solution) - DNA solution) of calf thymus DNA and its DES complexes in aqueous solution at neutral pH with different drug concentrations in the region of 1800 to 600 cm\(^{-1}\).

FIG. 2. Intensity ratio calculation for several DNA in-plane vibrations at 1717 cm\(^{-1}\) (G and T), 1663 cm\(^{-1}\) (A and T), 1609 cm\(^{-1}\) (A), 1492 cm\(^{-1}\) (C and G), and 1222 cm\(^{-1}\) (PO\(_2\) stretch) as a function of drug concentration (DES/DNA (phosphate) molar ratios).
Different drug concentrations in the region of 1800 to 600 cm⁻¹ and its DES complexes in aqueous solution at neutral pH with 1286, 1083, and 1046 cm⁻¹, resulting in an increase in the intensity of several DNA in-plane vibrations (33, 34). The reduction in the intensity and shifting of the Raman marker line at 838 cm⁻¹ (B-DNA) toward a lower frequency at 833 cm⁻¹ together with the appearance of a shoulder line at 820 cm⁻¹ (A-DNA) are also evidence for a major reduction of B-DNA structure in favor of A-DNA upon drug interaction (Fig. 3, r = 1). However, the reduction in the intensity and shifting of the Raman marker line at 838 cm⁻¹ (B-DNA) toward a lower frequency at 833 cm⁻¹ together with the appearance of a shoulder line at 820 cm⁻¹ (A-DNA) are also evidence for a major reduction of B-DNA structure in favor of A-DNA upon drug interaction (Fig. 3, r = 1). However, the reduction in the intensity and shifting of the Raman marker line at 838 cm⁻¹ (B-DNA) toward a lower frequency at 833 cm⁻¹ together with the appearance of a shoulder line at 820 cm⁻¹ (A-DNA) are also evidence for a major reduction of B-DNA structure in favor of A-DNA upon drug interaction (Fig. 3, r = 1). It should be noted that, although the PO₂ band at 1222 cm⁻¹ shifted toward a higher frequency at 1232 cm⁻¹ (IR spectra) and the Raman line at 833 cm⁻¹ (phosphodiester mode) moved toward a lower frequency at 820 cm⁻¹ on drug complexation, no major drug-PO₂ interaction occurred in these DES-DNA complexes. Evidence for this comes from no considerable intensity variations of the backbone PO₂ band at 881 cm⁻¹ upon DES intercalation (Fig. 2). The major spectral shiftings observed for the phosphate vibrations are due to the alterations of the DNA secondary structure during drug complexation.

Additional evidence for drug-DNA complexation comes also from the spectral changes of some of the DES in-plane vibrational frequencies. The shifts of the infrared bands of free DES at 1513 cm⁻¹ to 1510 cm⁻¹, 1370 cm⁻¹ to 1375 cm⁻¹, 1257 cm⁻¹ to 1260 cm⁻¹, 1045 cm⁻¹ to 1037 cm⁻¹, 884 cm⁻¹ to 871 cm⁻¹, and 836 cm⁻¹ to 838 cm⁻¹ upon DNA interaction are characteristic of drug-DNA complexation (Fig. 1). Similarly, the shifts of the free DES Raman lines at 1457 cm⁻¹ to 1454 cm⁻¹ and at 1048 cm⁻¹ to 1050 cm⁻¹ are related to drug-DNA complexation (Fig. 3). A strong line at 881 cm⁻¹ in the Raman spectra of free DES, which was observed at 879 cm⁻¹ in the spectra of drug-DNA complexes, is due to ethanol vibration, and it is eliminated from difference spectra for clarity (Fig. 3).

Comparison with Other DNA Intercalators—The infrared spectra of DES-DNA complexes were compared with those of strong DNA intercalators (12, 22, 37, 38) such as ethidium bromide, acridine orange, and methylene blue, recorded in our laboratory, and the results are compared here accordingly. A strong dye-PO₂ interaction was observed for ethidium bromide, acridine orange, and methylene blue upon intercalation (external binding with the backbone phosphate groups) due to major intensity variations of the phosphate vibration at 1222 cm⁻¹. The DES-PO₂ interaction was not observed in these drug-DNA complexes. Major DES interaction with the thymine oxygen atom occurred (groove binding) upon drug intercalation, while such interaction was not observed in the case of dye-DNA intercalation (12, 22, 38). Although the dye-DNA intercalation resulted in a minor local alteration of the backbone phosphate geometry, no major departure from B-DNA structure was observed upon dye complexation. However, the DES-DNA binding resulted in a major reduction of B-DNA structure in favor of A-DNA. The dye-DNA complexation was not mainly sequence-specific (38), while DES interaction was with the A-T bases at low drug concentration and progressed toward G-C base pairs (external binding) at a high drug content.

In conclusion, the vibrational spectroscopic results presented here, for the first time, clearly show that DES intercalates along the A-T-rich region in a way that the aromatic parts are inserted into the A-T base pairs, while the OH groups stretch externally toward the thymine oxygen atom (groove binding).
At high drug concentration, groove binding also occurs with G-C donor sites, which results in a partial helix opening. No drug-PO$_2$ interaction was observed, while a partial reduction of B-DNA structure occurred in favor of A-DNA.

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