Physicochemical Studies on the Interaction of Irehdiamine A with Bihelical DNA†

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ABSTRACT: The binding of two diamino steroids, irehdiamine A and dihydroirehdiamine A, to double-stranded DNA has been studied in the presence of various NaCl concentrations up to 0.20 M. As shown by Mahler et al. [Mahler, H. R., Goutarel, R., Khuong-Huu, Q., and Truong-Ho, M. (1966), *Biochemistry 5,* 2177], these steroids cause DNA stabilization for a number of bound drugs per phosphate residue smaller than about 0.2 and DNA destabilization above this value. The binding is reversible as shown by the complete dissociation of the complex observed upon addition of mineral or organic cations. The binding equilibrium was studied by preparative ultracentrifugation and the parameters relative to the stabilized complex were determined. The binding constant of irehdiamine A decreases as a function of the salt concentration according to the equation: $\log K = 2.19 - 1.49 \log [NaCl]$. The binding constant of dihydroirehdiamine A is slightly smaller than that of irehdiamine A. Entropy is the predominant component of the free-energy change of the reaction. The DNA–steroid binding constants were also measured by analysis of the competition between ethidium and steroids for the DNA binding. The steroid-induced alterations of the DNA UV absorption spectrum were studied: the steroids cause a maximum hyperchromicity at 260 nm of the DNA of about 18%. Especially at the higher ionic strengths, the hyperchromicity is not a linear function of the amount of bound steroid, but appears to be induced cooperatively. The superhelical turns of the closed circular DNA molecule of phage PM2 are removed for a bound steroid/phosphate ratio of 0.17 in a solvent containing 0.05 M NaCl. This unwinding is presumably due to the opening of 7% of the DNA base pairs. Evidence for the opening of the DNA base pairs is provided by the measurement of the rate of reaction of the bases with formaldehyde in the presence of irehdiamine A. Fast reannealing of the DNA denatured in the presence of steroid at high concentration was observed, which suggests the existence of cross-links between the two DNA strands. Finally, it is proposed that the stabilizing site of binding lies in the DNA minor groove.

The expression and the control of the biological functions of DNA involve the reversible binding of a large variety of polypeptides and complex proteins. Smaller molecules such as antibiotics and dyes are also able to bind to DNA in vitro as well as in vivo, eventually competing with the DNA-binding proteins. The free-energy change associated with the formation of these associations varies from a few kcal/mol up to about 16–18 kcal/mol. Most of these ligands carry positively charged groups which interact with DNA phosphates. Consequently, the electrostatic contribution to the binding free-energy change is usually predominant, and in solutions containing a high salt concentration the binding is prevented or greatly reduced. When interacting with a ligand, the DNA may eventually undergo a structural transition such as the local unwinding occurring in the intercalation of planar dyes between adjacent base pairs of the helix (Lerman, 1961). A local unwinding probably not due to intercalation but sufficient to cause the disruption of several base pairs of the DNA of phage λ upon binding of *Escherichia coli* RNA polymerase has also been observed by Saucier and Wang (1972). From the recent evaluation of the unwinding angle of ethidium (Wang, 1974), the unwinding caused by RNA polymerase corresponds to the opening of seven or eight base pairs. Some ligands, such as spermine which presumably binds in the small groove of the DNA double helix (Liquori et al., 1967), do not appreciably alter the DNA structure (Waring, 1970). Conversely, the binding of histones to DNA unwinds it, as shown by Germond et al. (1975) who found that the DNA fragment contained in one nucleosome is unwound by about 360°. In addition to the charged compounds cited above, DNA can also bind nonionic, nonhydrosoluble molecules of great biological interest like steroid hormones (Cohen et al., 1969) and carcinogenic hydrocarbons (Ts'o et al., 1969), but the very low level of binding obtainable has precluded the search for any alteration induced by these compounds in the DNA structure. Due to their solubility in water, steroidal alkaloids first studied by Mahler and Dutton (1964) were shown to be able to bind to DNA to such an extent that their effects on the DNA structure are easily detectable. As shown by these authors, the interaction of cyclobuxine D with DNA results in two opposite effects: at a low steroid/phosphate ratio stabilization against thermal denaturation of the DNA is observed, whereas at a high steroid/phosphate ratio the DNA is destabilized. In addition, a significant alteration of the DNA UV absorption spectrum was observed. Two other steroidal alkaloids, irehdiamine A and its dihydrogenated derivative, bearing less substituents bind to DNA with a higher affinity and have a similar effect as cyclobuxine D on its physical properties (Mahler et al., 1966; Saucier et al., 1968). It was subsequently shown using polyribonucleotides that IDA[1] does not exhibit any base specificity (Lefresne and Jacquemin-Sablon, 1969) but is able upon binding to bring poly(riboinosinic acid) into an ordered structure (Lefresne et al., 1967). The essential characteristics

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[1] Abbreviations used are: IDA, irehdiamine A; H_2IDA, dihydroirehdiamine A; EB, ethidium bromide; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; T_m, temperature of the DNA half-transition determined at 260 nm.

of the DNA–IDA interaction have been reviewed by Mahler and Green (1970). Most interestingly, IDA has been shown to remove and reverse the supercoiling of closed circular ϕX174 replicative form DNA as well as PM2 DNA (Waring, 1970; Waring and Chisholm, 1972). In order to assess the amount of bound steroid causing a change in any observed physical property of DNA, a detailed analysis by several independent methods of the stoichiometry of IDA and H_2IDA binding to calf thymus DNA has been carried out, and the UV absorption spectra of these complexes have been quantitatively analyzed. The results obtained suggest that the DNA small groove is the preferential site of binding of these steroids for the stabilizing interaction. Several properties of destabilized DNA have also been studied, and destabilization is shown to be correlated to DNA conformational changes characterized by the opening of complementary base pairs. Measurement of the rate of reaction of DNA bases with formaldehyde shows that the disruption of the base pairs is induced cooperatively by the steroid. In conclusion, it is proposed that the unwinding of superhelical DNA previously reported is due to the opening of a fraction of the DNA base pairs as observed in the alkaline titration of superhelical DNA (Vinograd et al., 1968; Wang, 1974).

Experimental Section

Materials

Steroids. Irehdiamine A (pregn-5-ene-3β,20α-diamine), prepared according to Truong-Ho et al. (1963), was kindly supplied by Dr. Goutarel. It is readily soluble in water as the dihydrochloride (more than 0.02 M) or as the diacetate (more than 0.08 M), whereas IDA phosphate and carbonate are practically insoluble. However, the solubility of the dihydrochloride is greatly reduced in concentrated salt solutions. UV absorbance of IDA in aqueous solution is very low, and in most cases negligible compared to that of DNA (ϵ 14.0 M^{-1} cm^{-1} at 260 nm). Dihydroirehdiamine A (5α-pregnane-3β,20α-diamine) was prepared by hydrogenation of IDA (Truong-Ho et al., 1963). Dihydrochloride and diacetate are hydrosoluble, and the UV absorption spectra of these salts are quite similar to the IDA spectrum. Tritium-labeled dihydroirehdiamine A (15.3 Ci/mol) was prepared by Dr. Nam using tritium instead of hydrogen and purified by thin-layer chromatography.

Nucleic Acids. Calf thymus DNA was used throughout this work unless otherwise stated; it was prepared either according to Kay et al. (1952) or Aubin et al. (1963). Further purification was achieved by pancreatic ribonuclease treatment according to Marmur (1961), followed by phenol extraction and dialysis against 0.5 M NaCl. The molecular weight, estimated by ultracentrifugation, was about 8×10^6. Spectral analysis according to Hirschman and Felsenfeld (1966) was performed on all DNA samples. The extinction coefficient determined by this method (6430 $\pm$ 20 M^{-1} cm^{-1}) is in agreement with the value reported by these authors. Low-molecular-weight DNA was produced by ten 1-min cycles of sonication with a Branson S-125 Sonifer. The DNA solution (1 mg/mL) in 0.02 M phosphate buffer, pH 7.0, was saturated with nitrogen before each sonication step, and finally phenol extracted and dialyzed as above. This treatment resulted in a molecular weight drop to 3.0×10^5 with no noticeable change in T_m and absorption spectrum. Heat-denatured DNA was prepared by heating a calf thymus DNA solution at 100 °C in a sealed vial; it was then quickly cooled to 0 °C. Covalently closed circular DNA from phage PM2 was prepared as described by Le Pecq (1971). *Micrococcus luteus* DNA was prepared according to Marmur (1961).

Chemicals. Ethidium bromide was from Boots Pure Drug Co., Ltd., Nottingham, U.K. Phenol was distilled under nitrogen, saturated with 0.001 M EDTA, 0.01 M Tris-HCl (pH 7.5), and stored at −20 °C. Pancreatic ribonuclease from Worthington Biochemical Corp., Freehold, N.J., was heated for 10 min at 100 °C in water to inactivate any contaminating deoxyribonuclease.

Methods

Ultracentrifugation Analysis. Binding equilibria were analyzed by a centrifugation method following the basic principles of Steinberg and Schachman (1966); DNA–steroid complexes were pelleted down by a 15-h run at 37×10^3 rpm in the rotor 40-3 of a Spinco L2 preparative ultracentrifuge. The steroid concentration determined in the supernatant can be taken as equal to the free alkaloid concentration in the initial mixture under two conditions: (1) No sedimentation of the free ligand must occur during the run. This point has been checked by running simultaneously a control sample lacking DNA. (2) The binding equilibrium must be independent of hydrostatic pressure changes. This point has been checked by measuring the amount of $^3H_2$IDA pelleted with DNA under identical conditions but with different column heights. A slight but significant increase in the amount of bound steroid was observed as a result of increasing the volume of the sample from 2 to 5 mL. However, since the variation in the amount of bound steroid did not exceed 5%, this effect was not taken into account. The residual absorbance at 260 nm indicated that less than 1 to 2% of the DNA remained in the supernatant after centrifugation. The amount of bound steroid was therefore obtained by the difference between the initial steroid concentration and the concentration in the supernatant measured by a colorimetric method as described. The use of labeled $^3H_2$IDA enabled us to make a direct measurement of the bound steroid in the pellet: after carefully withdrawing the supernatant, the pellet was dissolved in 0.5 mL of 0.1 N sodium hydroxide at 60 °C for 10 min and neutralized with 0.5 mL of 1 N hydrochloric acid, and 0.1-mL aliquots were assayed for radioactivity.

Determination of the Binding Parameters. In the absence of mutual interaction between bound ligands and assuming all the DNA binding sites are equivalent, the binding equilibrium can be adequately described by one of the following equations (Scatchard, 1949):

$$r/S_F = K(n - r) \quad (1)$$

$$r = nKS_F/1 + KS_F \quad (2)$$

where S_F is the free ligand molar concentration, r the number of bound ligand molecules per nucleotide, n the limit value of r at saturation, and K the apparent binding constant. From a measurement of S_F and r, the binding parameters n and K can be derived using eq 1. Given n and K, the nucleotide molar concentration P, and the total steroid molar concentration $S_T = S_F + Pr$, the binding ratio r can be calculated as the smaller than n root of the following equation derived from eq 1 or 2:

$$Pr^2 - (nP + S_T + K^{-1})r + nS_T = 0 \quad (3)$$

Miscellaneous. Absorbance measurements were carried out using a Zeiss PMQ II spectrophotometer. Spectrophotometric titration curves were obtained by addition of the steroid solution with a micrometer syringe to the DNA solution in a 2-cm path-length cell equipped with a magnetic stirring bar. The data were corrected for dilution and, whenever necessary, for the specific absorbance of the steroid. Differential spectra were obtained with a Cary 15 double-beam spectrophotometer. The DNA thermal transitions were analyzed by absorption mea-

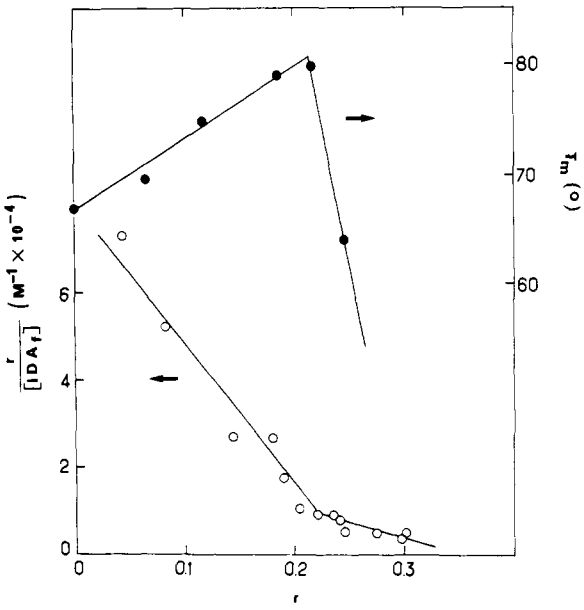

FIGURE 1: DNA-IDA binding equilibrium in 0.01 M NaCl solution at 20 °C (○). Solutions containing 1×10^{-4} M P-DNA and various concentrations of IDA up to 1×10^{-4} M were analyzed by ultracentrifugation. The temperature of half-transition of DNA determined by absorption spectrophotometry at 260 nm in the same solvent in the presence of IDA is plotted as a function of r determined at 20 °C (●).

surements at 260 nm using a thermostated cell holder. The temperature was measured inside the cell with a thermocouple. The rate of reaction of the DNA bases with formaldehyde was measured at room temperature by spectrophotometry at 275 nm as described by Haselkorn and Doty (1961) using a solvent containing 0.01 M sodium cacodylate, pH 7.0. Spectrofluorimetric analysis of ethidium binding to DNA was performed according to Le Pecq and Paoletti (1967) with the fluorescence attachment Zeiss ZFM 4. Excitation and emission wavelengths were respectively set at 546 and 590 nm. Sedimentation coefficients were determined using a Spinco Model E analytical ultracentrifuge at 39 450 rpm at 20 °C; 30-mm cells were used with a DNA concentration of 10 μg/mL. Molecular weights were derived using the relation of Crothers and Zimm (1965). The DNA buoyant density was measured in CsCl gradient at 25 °C at 44 770 rpm. Viscometry measurements were carried out at 20 °C using a low-shear capillary viscometer as described (Saucier et al., 1971). To determine IDA and H_2IDA concentrations, the amine functions of the steroids were reacted with 2,4,6-trinitrobenzenesulfonic acid, and the product of the reaction was measured by spectrophotometry (Mokrasch, 1967). No interference was found either with DNA or with free 5′-deoxyribonucleotides. Radioactive counting of ^3H_2IDA was performed in a Tricarb liquid scintillation spectrometer using a dioxane-based scintillation mixture containing per liter 4 g of PPO, 0.2 g of Me_2POPOP, 60 g of naphthalene, and 100 mL of ethoxyethanol. Molecular models of bihelical DNA and IDA were built at the scale of 1.25 cm/Å using CPK elements obtained from Schwartz Bioresearch Inc.

Results

DNA–IDA Binding Analysis by Ultracentrifugation. Since the IDA extinction coefficient at 260 nm is small, the DNA thermal transitions can be analyzed at this wavelength at different r values. As shown in Figure 1, T_m increases linearly as a function of r in the range $r < 0.21$, the DNA destabilization becoming apparent for $r > 0.21$. Binding equilibrium measurements carried out as described in the Experimental Section are plotted in Figure 1 showing that $r/[IDA_f]$ decreases linearly as a function of r for r values up to about 0.20 where the isotherm exhibits a marked change of slope. These observations can be accounted for by the existence of two types of complexes (Mahler and Green, 1970). The binding parameters of the stabilized (type I) complex were derived from the data obtained by ultracentrifugation, taking into account only r values below 0.20 (Table I). It was observed that in this range the binding equilibrium is unaffected by temperature changes between 4 and 30 °C. Spectrophotometric analysis of the interaction at different temperatures confirmed this observation (see below). As shown in Figure 2, log nK decreases linearly as a function of log [NaCl]. Since log n is constant, K is conveniently expressed as a function of the salt concentration of the medium by the following equation:

$$\log K = 2.19 - 1.49 \log [\text{NaCl} \qquad (4a)$$

The determination of the binding parameters at salt concentrations above 0.05 M was unreliable, since the bound fraction of steroid is decreased to such an extent that its evaluation by the difference between the initial steroid concentration and the steroid concentration in the supernatant is quite inaccurate. However, in the case of tritium-labeled H_2IDA where no such limitation existed, the linear dependence of log nK upon log NaCl was observed up to 0.2 M NaCl. This suggests that the relationship in eq 4a is similarly valid in the case of IDA up to this salt concentration.

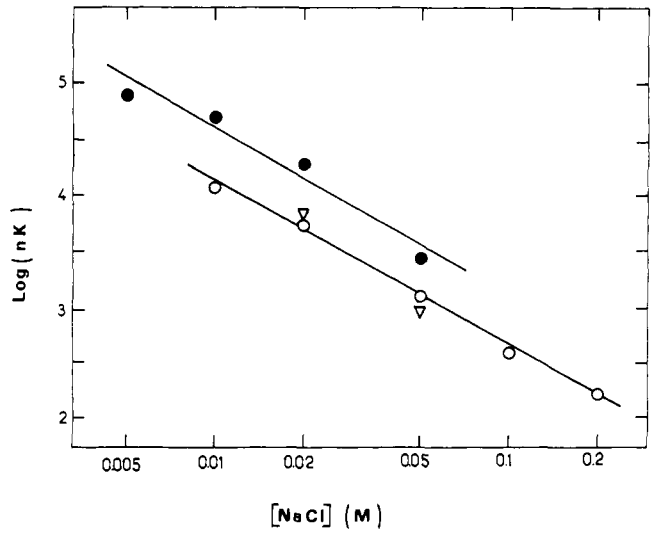

FIGURE 2: Effect of the salt concentration upon the DNA-steroid binding constants. Log (nK) is plotted as a function of NaCl concentration at 20 °C. K and n values for IDA (●) are taken from Table I; for H_2IDA, nK was either derived from the initial part of the binding isotherm (○) or from the complete isotherm (△) shown in Figure 3.

TABLE I: DNA-IDA Binding Parameters Determined by Ultracentrifugation at 20 °C.[a]

[NaCl] (M)	n	K (M^{-1})	$-\Delta G°$ (kcal/mol)[b]
0.005	0.29	2.6×10^5	7.4
0.01	0.27	1.8×10^5	7.2
0.02	0.23	8.1×10^4	6.7
0.05	0.28	9.7×10^3	5.4

[a] Values relative to the linear part of the Scatchard plot (stabilizing binding sites). Each figure is the average of two to four independent determinations. [b] $\Delta G° = -RT \ln k$ is the free-energy change of the binding reaction.

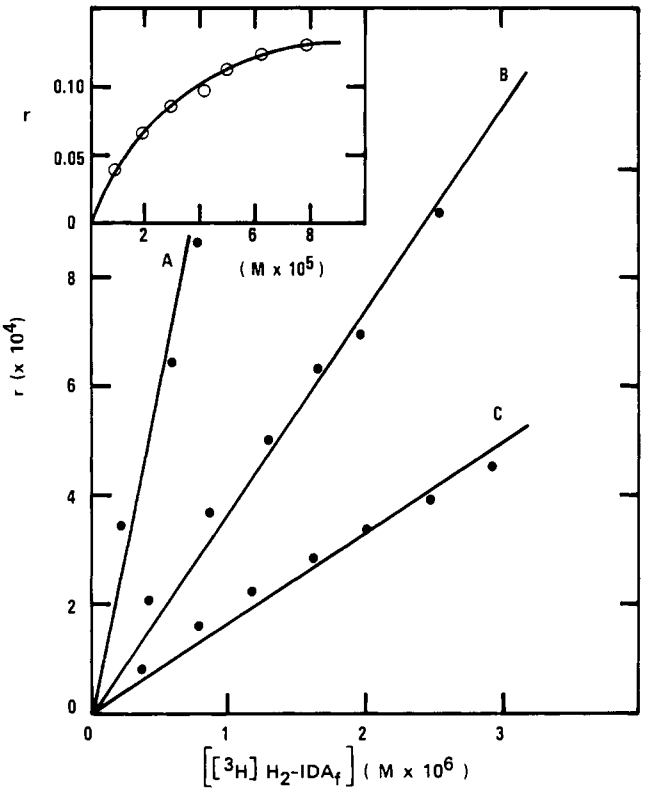

FIGURE 3: Detail of the initial part of the DNA-^3H_2IDA binding isotherm analyzed by ultracentrifugation at 20 °C: (A) 2.5×10^{-4} M P-DNA in 0.05 M NaCl; (B) 4.16×10^{-4} M P-DNA in 0.1 M NaCl; (C) 7.26×10^{-4} M P-DNA in 0.2 M NaCl. The isotherm shown in the inset was obtained with 1×10^{-4} M P-DNA in 0.02 M NaCl.

DNA-^3H_2IDA Binding Analysis by Ultracentrifugation. More accurate data were obtained when the amount of bound steroid was determined directly in experiments carried out using a tritium-labeled steroid. In addition to better accuracy, the use of ^3H_2IDA permits an analysis of the equilibrium at a very low binding ratio, as shown in Figure 3 where r values smaller than 0.001 have been measured as a function of S_F. These data were obtained at a relatively high salt concentration; under these conditions, no binding could have been detected for IDA due to the above-cited technical limitations. The slope of the binding isotherm plotted according to eq 2 is equal to nK, when $r \ll n$. Using the nK values derived from the data shown in Figure 3, a linear dependence of log nK is observed as a function of log NaCl (Figure 2) as in the case of IDA and with the same slope value:

$$\log K = 1.79 - 1.47 \log [\text{NaCl}] \qquad (4b)$$

Since some cooperativity was observed in the displacement of ethidium bromide by IDA as well as H_2IDA, and in the spectral changes induced by these steroids upon binding to DNA, the question arises as to whether the binding itself is cooperative. As shown in Figure 3, no cooperativity is apparent in the initial part of the binding isotherm; furthermore, data plotted in Figure 2 indicate that nK values determined in the initial part of the binding isotherm are within experimental error equal to the nK values derived from the complete isotherm plotted according to eq 1. These observations indicate that the steroids binding to DNA are not cooperative.

Determination of the DNA-Steroid Binding Constants by Competition with Ethidium Bromide. The intercalation of ethidium into DNA was studied by fluorimetry as described by Le Pecq and Paoletti (1967) in the presence of IDA or

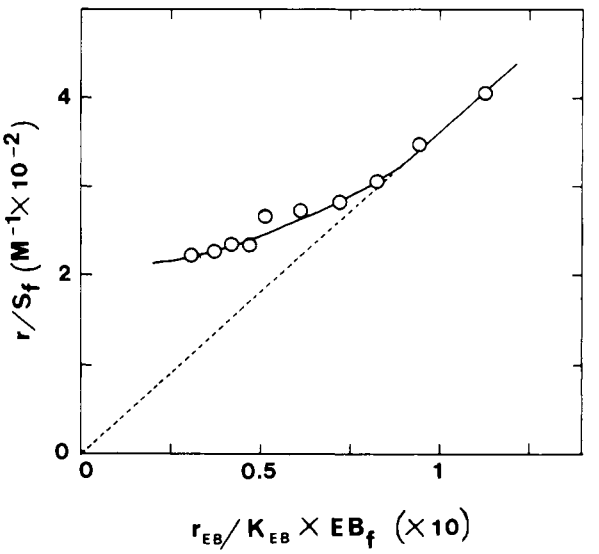

FIGURE 4: Binding measurement of ethidium to DNA in the presence of H_2IDA. The steroid was added to a solution containing 2.03×10^{-5} M P-DNA, 4.83×10^{-6} M EB in 0.05 M NaCl. The data are plotted according to eq 6, and the DNA-steroid binding constant is derived from the rectilinear part of the curve.

H_2IDA acting as competitors for the DNA binding. Measurements were performed in the presence of 0.05 M NaCl to avoid the nonintercalative binding of the dye. In the absence of steroid, the ethidium binding to DNA follows the equation of Bauer and Vinograd (1970), and competitive inhibition of the ethidium binding was observed in the presence of IDA or H_2IDA (data not shown). In order to determine the DNA-steroid binding constants, the steroids were added stepwise to a solution containing DNA and ethidium, and r_{EB} was determined as a function of the steroid concentration. Then the amount of bound steroid was calculated using the equation of McGhee and Von Hippel (1974) for two different species of ligands:

$$\frac{r_{EB}}{C_{EB}} = \left(\frac{K_{EB}}{2}\right)\left(\frac{(1 - 4r_{EB} - 4r)^2}{1 - 2r_{EB} - 2r}\right) \qquad (5)$$

where K_{EB} denotes the DNA-EB binding constant in the absence of steroid and C_{EB} the free EB concentration in the mixture. The DNA-EB and DNA-steroid binding constants are related by the following equation:

$$\left(\frac{1}{K}\right)\left(\frac{r}{S_F}\right) = \left(\frac{1}{K_{EB}}\right)\left(\frac{r_{EB}}{C_{EB}}\right) \qquad (6)$$

Therefore it is expected that r/S_F is proportional to r_{EB}/C_{EB}. The data plotted in Figure 4 show that this is the case only for the higher r/S_F values. Taking these values into account, the K values are found respectively equal to 9.0×10^3 M^{-1} for IDA and 3.6×10^3 M^{-1} for H_2IDA. Since the binding constants calculated with the excluded site model are about half the values obtained with the independent site model (Bauer and Vinograd, 1970), these results are in reasonable agreement with the results obtained by other methods. The deviation from eq 6 observed in Figure 4 at high steroid concentration and low r/S_F suggests that a cooperative displacement of EB by the steroid molecules is occurring for a number of steroids bound per phosphate residue larger than about 0.09.

Effect of Steroid Binding on the DNA Absorption Spectrum. Mahler et al. (1968) have reported that the UV absorbance of double-stranded DNA increases upon IDA binding. This observation was extended by analyzing differential

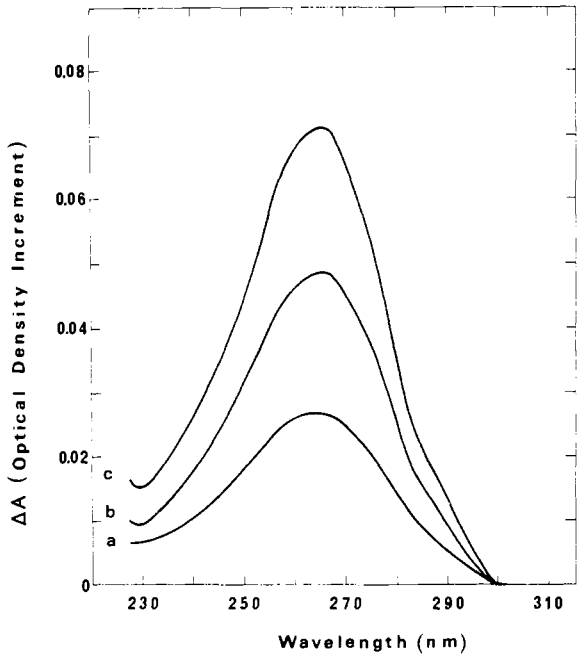

FIGURE 5: Differential spectra of DNA-IDA complexes at different r values. Measurements were performed in 1-cm cells with a Cary 15 spectrophotometer using the 0.1 OD scale. Both reference and sample mixtures contained 10^{-4} M P-DNA in 0.02 M NaCl. IDA was added to the sample mixtures so as to give r values respectively equal to 0.073 (a), 0.122 (b), and 0.188 (c).

spectra of the complex at various binding ratios vs. free DNA. As shown in Figure 5, the hyperchromic effect extends over the whole absorption band of DNA with a maximum centered at 265 nm independently of the r value. From these data, the possibility that the absorbance increment results from the light diffusion by aggregated DNA fibers is ruled out, since in that case the increment would be proportional to λ^{-4} according to Rayleigh's law. Furthermore, the absence of an isosbestic point suggests that the changes observed are due to a perturbation of the DNA absorption spectrum rather than a shift of the steroid far-UV spectrum. Since it was found that absorbance increments measured simultaneously at 260 and 280 nm are proportional (data not shown), it was inferred that the steroid is randomly distributed along the DNA molecule and exhibits no selective affinity for AT or GC base pairs whose 260/280 extinction coefficients ratios are quite different. Another consequence of this observation is that a single absorbance measurement at one wavelength is sufficient to determine the amount of bound steroid. Therefore, a series of measurements of absorbance increments (ΔA) was performed at 260 nm as a function of the steroid concentration at various salt concentrations (Figure 6). The DNA-steroid binding parameters were derived from these data as shown below.

In Figure 7, the correlation between ΔA and r calculated from ultracentrifugation data using eq 3 has been studied for the binding of IDA to DNA in the presence of 0.02 M NaCl. For r values above 0.1, a linear relationship is observed as previously reported (Saucier et al., 1968).

$$\Delta A = \Delta\epsilon r P l \qquad (7)$$

where l is the light path length and $\Delta\epsilon$ the variation of the DNA molar extinction coefficient at 260 nm when 1 mol of steroid is bound to 1 mol of DNA nucleotide. From the data in Figure 7, $\Delta\epsilon$ is found equal to 4.55×10^3 M^{-1} cm^{-1}. The deviation from linearity at low r values indicates that $\Delta\epsilon$ is not constant in this range. The increase of $\Delta\epsilon$ as a function of r is

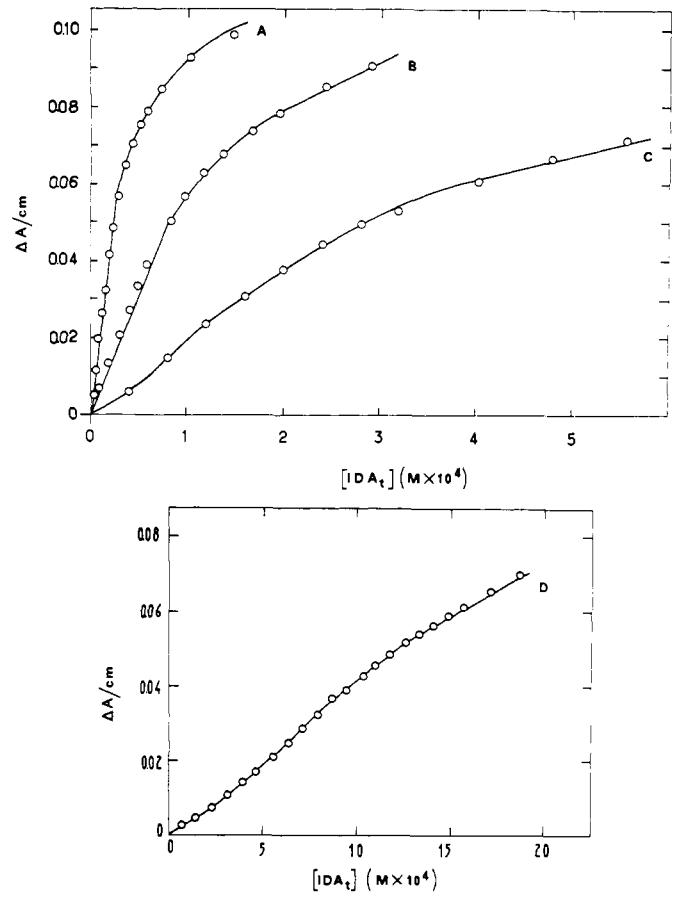

FIGURE 6: Spectrophotometric titration of DNA by IDA. Measurements were performed at 260 nm in 2-cm light-path cells. All solutions contained 10^{-4} M P-DNA and varying concentrations of IDA. The results obtained at four different NaCl concentrations are shown: 0.02 M (A); 0.05 M (B); 0.01 M (C); 0.2 M (D).

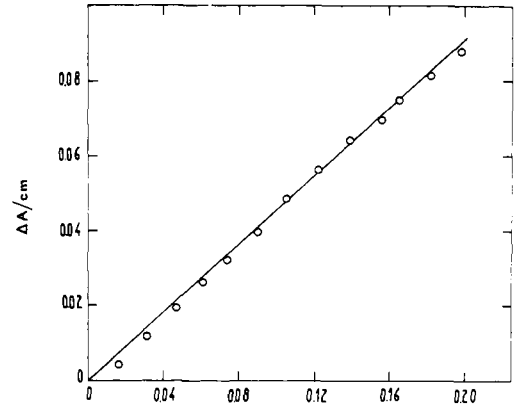

FIGURE 7: Relationship between the hyperchromicity and the amount of bound IDA. ΔA was measured under the conditions of Figure 6 (0.02 M NaCl) and r was calculated using eq 3.

more marked at high salt concentration, as shown by the sigmoidal shape of the curves $\Delta A = f$[IDA] plotted in Figure 6.

Spectrophotometric Analysis of the Binding Equilibrium. Since accurate spectrophotometric measurements are readily obtained (Figure 6), a method was sought to derive the DNA-steroid binding parameters directly from such data. For that purpose, it was assumed that the steroid binding follows eq 3 and that the hyperchromicity is proportional to the amount of bound steroid as expressed in eq 7. At saturation of the DNA

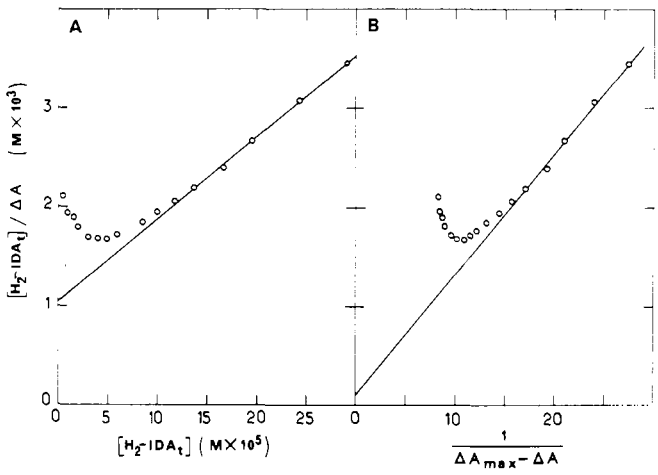

FIGURE 8: Spectrophotometric determination of the DNA-H_2IDA binding constant. Measurements were carried out under the same conditions as described in Figure 6 with 10^{-4} M P-DNA in 0.05 M NaCl (ΔA is the optical density increment for a 1 cm path length). The data are plotted in panel A according to eq 9, giving an evaluation of ΔA_{max}. In panel B, this value of ΔA_{max} is used to plot the data according to eq 8, allowing an evaluation of K.

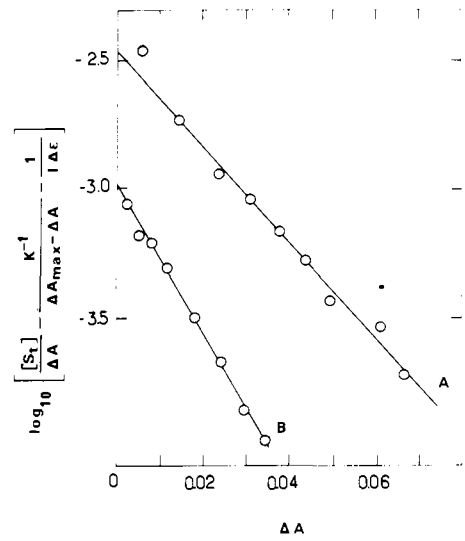

FIGURE 9: Spectrophotometric analysis of DNA interaction with IDA and H_2IDA. Data were plotted according to eq 11. (A) DNA-IDA in 0.1 M NaCl; (B) DNA-H_2IDA in 0.05 M NaCl (data from Figure 8).

TABLE II: Spectrophotometric Analysis of DNA-Amino Steroid Interaction.[a]

[NaCl] M	DNA-IDA K (M^{-1})[b]	DNA-IDA $n\Delta\epsilon$ (M^{-1} cm^{-1})[c]	DNA-H_2IDA K (M^{-1})[b]	DNA-H_2IDA $n\Delta\epsilon$ (M^{-1} cm^{-1})[c]
0.01	9.1×10^4	1.23×10^3	5.3×10^4	1.38×10^3
0.02	3.6×10^4	1.19×10^3	2.4×10^4	1.24×10^3
0.05	1.2×10^4	1.20×10^3	8.2×10^3	1.21×10^3
0.10	2.9×10^3	1.20×10^3	1.3×10^3	1.58×10^3

[a] Measurements were carried out as described in the caption of Figure 6. [b] The binding constant K was determined by plotting the data according to eq 8 as shown in Figure 8B. [c] $n\Delta\epsilon = \Delta A_{max}/P$ was determined by plotting the data according to eq 9 as shown in Figure 8A.

binding sites, this expression may be written $\Delta A_{max} = \Delta\epsilon nPl$. Combining this relation with eq 3 and 7 one obtains:

$$\frac{S_T}{\Delta A} = \left(\frac{1}{K}\right)\left(\frac{1}{\Delta A_{max} - \Delta A}\right) + \frac{1}{l\Delta\epsilon} \quad (8)$$

$$\frac{S_T}{\Delta A} = \frac{S_T}{\Delta A_{max}} + \frac{1}{K\Delta A_{max}} + \frac{1}{l\Delta\epsilon}\left[1 - \frac{\Delta A}{\Delta A_{max}}\right] \quad (9)$$

In order to verify the validity of eq 8, the numerical value of ΔA_{max} has to be known, but it cannot be obtained through a direct measurement due to the interference of the destabilizing effects observed at high steroid concentration. However ΔA_{max} can be derived by plotting the data according to eq 9. The last term of the second member of this equation is small ($\leq 2.5 \times 10^{-4}$), compared to $S_T/\Delta A$ which is always larger than 1.6×10^{-3} in the case of Figure 8A, especially at high steroid concentration. Therefore, at high steroid concentration eq 9 can be approximated by:

$$\frac{S_T}{\Delta A} = \frac{S_T}{\Delta A_{max}} + \frac{1}{K\Delta A_{max}} \quad (10)$$

Consequently, when the data are plotted according to eq 9, they fit at high steroid concentration a straight line which provides an accurate determination of ΔA_{max} (Figure 8A). Examination of the results presented in Table II reveals that ΔA_{max} is within experimental error independent of the ionic strength. Since it has been shown that n is independent of the ionic strength, it follows that $\Delta\epsilon$ is also independent of the ionic strength. The hyperchromicity at 260 nm of the steroid saturated DNA, calculated from the average ΔA_{max} value reported in Table II, is 18% for IDA and 19% for H_2IDA.

Using the ΔA_{max} values obtained by this procedure makes it possible to plot the data according to eq 8, but a large deviation from this equation appears for low ΔA values (Figure 8B). Under these conditions, as shown in Figure 9, the data follow the empirical equation:

$$\frac{S_T}{\Delta A} - \left(\frac{1}{K}\right)\left(\frac{1}{\Delta A_{max} - \Delta A}\right) - \frac{1}{l\Delta\epsilon} = \exp(\alpha - \beta\Delta A)$$

There are two possible explanations to account for by that result: the steroid binding might be cooperative, but this has been ruled out by the results presented above, or ΔA is not proportional to r, as already suggested by the data plotted in Figure 7 for low ΔA values. This hypothesis is discussed below. The binding constants can be derived from the measurement of the slope of the linear part of the curves as plotted in Figure 8B. Results of such determinations presented in Table II show a decrease of K as a function of the ionic strength with the same slope for both steroids. Furthermore, in agreement with the results obtained previously, the IDA binding constant is found larger than the H_2IDA binding constant by a factor equal in the average to 1.7. Finally, it should be pointed out that n can also be independently derived from the spectrophotometric measurements, since a plot of the data according to eq 8 gives the value of $l\Delta\epsilon = \Delta A_{max}/nP$. However, in order to determine ΔA_{max} according to the method proposed above, $1/l\Delta\epsilon$ must be small compared to $S_T/\Delta A$. As a result, the n values derived from such measurements are inaccurate but their order of magnitude is nevertheless compatible with the ultracentrifugation results.

Influence of Some Physicochemical Factors on the DNA-Steroid Interaction. Since the binding constant decreases as a function of NaCl concentration, dissociation of the DNA-steroid complex is expected to occur upon raising the salt concentration of the medium, if the binding is reversible. As shown in Figure 10, the binding is indeed reversible, since ΔA tends toward zero when the NaCl concentration increases. Similarly, divalent cations such as Mg^{2+} and spermine displace IDA from DNA as shown in Figure 10. With spermine, the

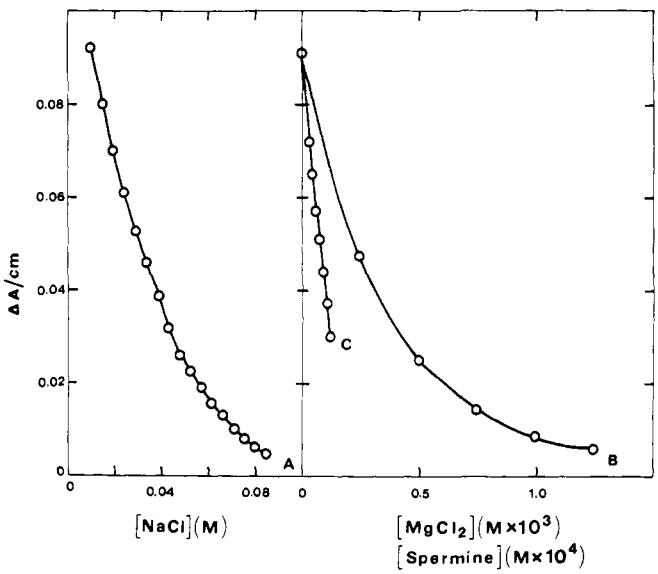

FIGURE 10: Effect of cations on the binding of IDA to DNA. To a solution containing 1×10^{-4} M P-DNA, 5×10^{-5} M IDA, and 1×10^{-2} M NaCl was added a concentrated solution of NaCl (A), $MgCl_2$ (B), or spermine hydrochloride (C). The optical density of the mixture was measured as a function of the cation concentration.

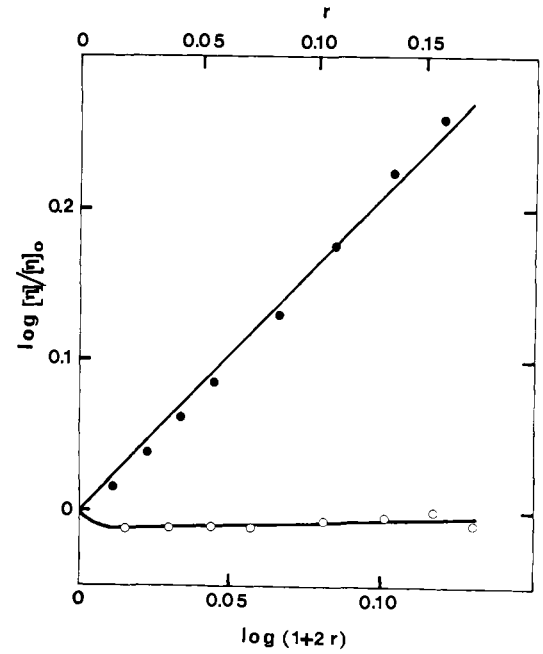

FIGURE 11: Effect of IDA on the length of sonicated DNA measured by viscometry. Measurements were performed in a capillary viscometer at 20 °C using sonicated DNA of molecular weight $\sim 3 \times 10^5$ in 0.05 M NaCl. Small volumes of concentrated solution of IDA were added and the flow time was measured as a function of IDA concentration (○). Equation 3 was used to calculate the amount of bound steroid. The same experiment was done with ethidium (●) which increases the DNA contour length according to the equation: $L/L_0 = 1 + 2r$. In ideal conditions $[n]/[n]_0 = (1 + 2r)^3$. The exponential factor is actually smaller than 3 (Saucier et al., 1971) and equal to 2.1 in the present case. Initial DNA concentration was 750 μg/mL in the measurements with IDA and 495 μg/mL in the measurements with ethidium.

displacement cannot be observed through completion due to the occurrence of DNA precipitation. However, it is noteworthy that spermine competes with IDA for DNA binding much more efficiently than $MgCl_2$ which is itself more efficient than NaCl. The effect of temperature has been studied in 0.05 M NaCl by measuring ΔA as a function of the IDA concentration at 25 and 45 °C. Identical results, within experimental error, were obtained at the two temperatures. K is therefore constant as a function of temperature, as already observed in the ultracentrifugation experiments. The enthalpy of the binding reaction is equal to zero, and the free-energy change of the reaction appears to be essentially entropic. A comparable result has been obtained by Le Pecq and Paoletti (1967) for the DNA-EB equilibrium at low salt concentration, an enthalpy contribution being detectable only above 0.1 M NaCl.

The effect of pH was also studied in 0.05 M sodium cacodylate buffer at pH 7.2, 6.2, and 5.2: ΔA was observed to decrease by no more than 5% at pH 5.2. This is probably related to the onset of acid titration of DNA at pH ~5.5 (Cox and Peacocke, 1957). On the other hand, the pK' of the two primary amines of IDA being about 9.6 (Mahler et al., 1968), all the steroid molecules can be considered as under the cationic form below pH 7.6. It is therefore concluded that from pH 5.5 up to about 7.6 the steroid binding is unaffected by pH changes. Spectrophotometric analysis of the interaction of IDA with DNA from *E. coli* (50% GC) and *Micrococcus luteus* (72% GC) gave comparable results, and this observation supports the notion that the steroid binding does not exhibit any base specificity.

Spectrophotometric measurements with low-molecular-weight DNA obtained by sonic irradiation of calf thymus DNA gave results similar to those obtained with 8×10^6 daltons DNA, but no detectable change was observed in the UV absorption spectra of free 5′-deoxyribonucleotides in the presence of IDA at high concentration.

Effect of IDA on the DNA Structure. IDA has been shown to unwind supercoiled DNA (Waring, 1970; Waring and Chisholm, 1972). However, this unwinding is not believed to be the result of intercalation of the steroid nucleus between two adjacent base pairs. Intercalation causes DNA elongation which in the case of ethidium is easily detected by viscometry using low-molecular-weight DNA fragments (Saucier et al., 1971). By contrast, as shown in Figure 11, IDA at a binding ratio of up to about 0.17 does not cause any detectable increase of the length of DNA fragments of molecular weight ca. 3×10^5. This conclusively shows that DNA unwinding is not due to the intercalation of the steroid between adjacent base pairs. In Figure 12 are plotted the results of hydrodynamic measurements carried out with covalently closed circular DNA extracted from phage PM2 in the presence of IDA. The PM2 DNA closed molecule has been shown previously to contain 31 negative superhelical turns at 20 °C in a solvent containing 0.05 M NaCl (Saucier et al., 1971). This estimation based upon a value of 12° for the unwinding angle of ethidium is in good agreement with measurements carried out in comparable conditions by Smit and Borst (1971) and Waring and Henley (1975). For each measurement of the sedimentation coefficient and the reduced viscosity of PM2 DNA, the r value was calculated using the binding parameters determined previously without taking into account the effect of the DNA superhelix free-energy change upon the steroid binding (Bauer and Vinograd, 1970). Since the average unwinding angle of IDA is only a fraction of the unwinding angle of ethidium, the resulting error on r is small. As shown in Figure 12, the PM2 DNA molecule contains zero superhelical turns for a steroid/DNA binding ratio equal to 0.17 ± 0.01. The good agreement between the two sets of measurements in which the DNA concentration was different by a factor of about 20 indicates that r has been accurately estimated at the equivalence

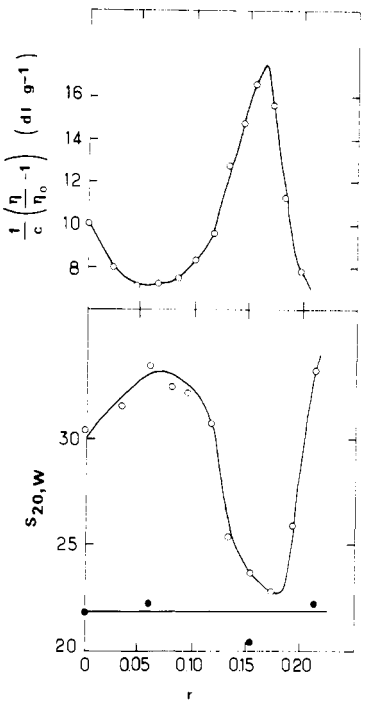

FIGURE 12: Unwinding of covalently closed circular PM2 DNA by IDA. Viscosity measurements were performed in a capillary viscometer at 20 °C in a solvent containing 0.05 M NaCl. The solution contained 5.0×10^{-4} M P-DNA, at least 95% of which was in the closed form. For the calculation of reduced viscosity, the DNA concentration was used without taking into account the amount of steroid bound to the DNA. Sedimentation coefficients of closed DNA (○) and nicked DNA (●) were determined in separate runs at 20 °C in the same solvent as above using solutions containing 3.1×10^{-5} M P-DNA. Equation 3 was used to calculate the amount of bound steroid.

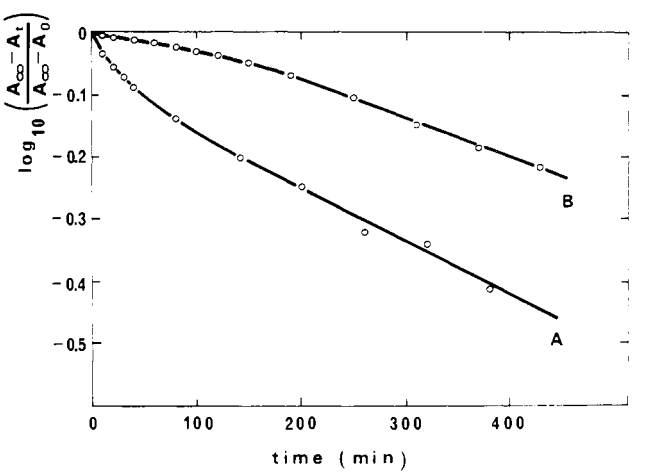

FIGURE 13: Effect of IDA on the reaction of DNA with formaldehyde. The optical density of mixtures containing 2×10^{-4} M P-DNA, 0.18 M formaldehyde, and 0.01 M sodium cacodylate, pH 7.0, was measured as a function of time; A_∞ was determined 48 h after the reaction was started. Curve A was obtained with heat-denatured DNA and curve B with native DNA in the presence of 1.5×10^{-4} M IDA.

point. For the titration of PM2 DNA with ethidium, the equivalence is observed for a binding ratio of 0.047 (Saucier et al., 1971); the unwinding angle of IDA is therefore 0.047/0.17 = 0.28 times the ethidium unwinding angle. Finally, in striking contrast with what is observed in the titration of circular DNA with ethidium, the curves shown in Figure 12 appear clearly asymmetrical in the region of the equivalence point. In this region, below and above the equivalence point, the intrinsic viscosity of a DNA-small molecule complex is a linear function of r, with the ratio of the slopes of the curve usually close to -1. This is indeed the case with ethidium and daunomycin (Saucier et al., 1971). However, with IDA this ratio is equal to -1.9 (Figure 12), which suggests that the number of superhelical turns of the closed PM2 DNA does not change linearly as a function of the number of bound IDA molecules.

Effect of IDA on the Rate of Reaction of DNA Bases with Formaldehyde. In the appropriate conditions, the rate of hydroxymethylation of the DNA bases by formaldehyde is slow enough so as to be conveniently measured by absorption photometry (Trifonov et al., 1967). Such a reaction can be used in principle to estimate the degree of base pairing in the DNA-IDA complex. A formaldehyde concentration has been selected where the rate of reaction at 25 °C of native bihelical DNA is practically equal to zero, whereas the rate of reaction in the same conditions of heat-denatured DNA is easily measurable. As shown in Figure 13, in the presence of 0.18 M formaldehyde, the reaction of heat-denatured DNA, after an initial period of about 100 min where the rate is fast, follows a first-order kinetics with a rate constant equal to 1.9×10^{-2} min^{-1}. The effect of IDA on the DNA structure has been studied by adding various amounts of amino steroid to a mixture containing native DNA and formaldehyde. In these conditions, the amine functions of IDA react with formaldehyde, making it unable to bind to DNA. The amount of IDA bound to DNA is therefore not constant during such an experiment, but decreases continuously. However, since the reaction of IDA with formaldehyde has no appreciable effect on the absorption of the solution at 275 nm, the measurement of the rate of reaction of the DNA bases is not perturbed by the reaction of the steroid. With IDA at the initial concentration of 5×10^{-5} M, the DNA is stabilized and the rate of reaction of the bases with formaldehyde is very slow and of the same order of magnitude as the rate observed in the absence of IDA. At the initial concentration of 1.0×10^{-4} M IDA, DNA is destabilized and, concurrently, a slight increment of the rate of reaction of the bases with formaldehyde is observed. This effect is tentatively attributed to the disruption of a small fraction of complementary base pairs. At the initial concentration of 1.5×10^{-4} M IDA, the complex has a T_m smaller than 25 °C and the DNA bases are readily accessible to formaldehyde, as shown by the data plotted in Figure 13. After a lag period of about 150 min, the reaction follows an apparent first-order kinetics with a rate constant of 1.5×10^{-2} min^{-1} quite comparable to the rate constant observed with heat-denatured DNA.

Reversibility of the Thermal Transition of the DNA-IDA Complex. The thermal transition profile of a DNA sample in the presence of IDA initially bound at a ratio of 0.28 and spectrophotometric measurements carried out from the end of the transition back to room temperature are shown in Figure 14. No appreciable change in optical density was detected during the cooling down of the solution, which suggests that conformational changes induced by raising the temperature are irreversible at a high IDA binding ratio. Calf thymus DNA, in the absence of IDA, renatures slowly and only partially in the conditions of the present experiment in agreement with Britten and Kohne's observations (1968). The same behavior is observed in the presence of IDA for a binding ratio smaller than 0.21. In the denatured complex obtained at $r >$ 0.21, IDA is still displaced when the salt concentration is raised. Data in Figure 14 show that by raising the salt concentration to 0.16 M, the DNA extinction coefficient is lowered to a level nearly equal to the native DNA value. Furthermore,

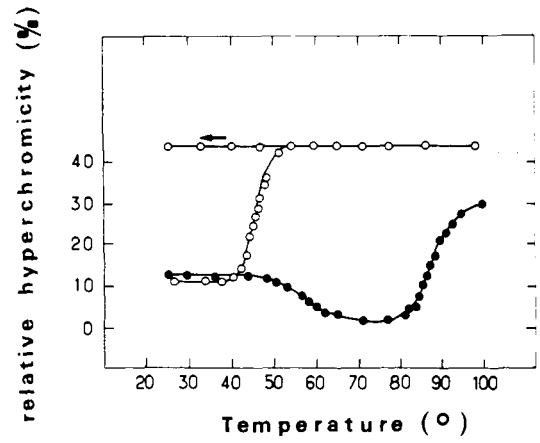

FIGURE 14: Thermal transition profile of DNA destabilized by IDA (r = 0.28 at 20 °C) in 0.01 M NaCl solution. The relative hyperchromicity is defined as $[(OD_t - OD_0)/OD_0]100$, where OD_0 is the optical density of the free native DNA at room temperature and OD_t the optical density of the complex at the indicated temperature. The measurements were first carried out as a function of temperature increasing from 25 to 98 °C (○); then after slow cooling to room temperature, the NaCl concentration of the solution was raised to 0.16 M, and measurements were carried out as a function of temperature in this solvent (●).

the structure obtained in these conditions closely resembles that of native DNA: the buoyant density in a cesium chloride density gradient at equilibrium is found equal within 0.001 g/mL to the buoyant density of native DNA. If the DNA is subjected to a second heating cycle (Figure 14), a small decrease of optical density corresponding to the reannealing of the few mismatched bases is first observed, then a transition is observed quite analogous to the thermal transition of native DNA in this solvent, as judged by the T_m and the hyperchromicity value. Contrasting with these observations, it was found that the addition of sodium chloride to DNA denatured in the presence of stabilizing amounts of IDA does not cause any structural change of the DNA.

Discussion

The complexes of IDA and H_2IDA with DNA were characterized by different physicochemical techniques. The binding equilibrium was studied by three independent methods, and the data were analyzed according to an independent binding-site model rather than the one-dimensional homogeneous lattice model of McGhee and Von Hippel (1974). The essential reason for this choice is that the number of lattice residues covered by one ligand, determined using the latter model, is not an integer, but is always larger than one and smaller than two. The isotherms corresponding to such a value are hardly distinguishable from the rectilinear isotherm of the classical Scatchard model (Scatchard, 1949). The curvature which is apparent on the binding isotherm of Figure 1 is therefore attributed to the existence of a distinct series of binding sites resulting in the formation of a destabilized complex. The binding constants relative to the stabilizing interaction, determined by the different methods, were generally found in good agreement, although in the case of IDA the analysis of the equilibrium is somewhat inaccurate, giving values significantly higher than the results obtained by spectrophotometry. Since no large effect of the hydrostatic pressure upon the equilibrium has been detected, it is assumed that a systematic error was made in the evaluation of the amount of bound steroid deduced from the measurement of the free steroid concentration in the centrifugation supernatant.

No cooperativity in the binding is detected by ultracentrifugation analysis. However, the hyperchromic effect induced by the bound steroid molecules is cooperative, especially at a high ionic strength and a low-binding ratio (<0.1), since in these conditions the results show that the hyperchromicity is not a linear function of the amount of bound steroid. Concurrently, at a binding ratio larger than about 0.09, IDA and H_2IDA appear to be able to displace ethidium from DNA in a cooperative fashion.

Different methods have been used to detect and characterize the DNA conformational changes induced by IDA at a high steroid/phosphate ratio. In spite of the fact that IDA unwinds supercoiled DNA, its intercalation between adjacent base pairs is ruled out for the main reason that the steroidal nucleus lacks the planar structure required for this process. In addition, there is no significant change of length of the DNA molecule in the presence of IDA, in contrast with the effect observed with DNA intercalating drugs. Waring and Chisholm (1972) have shown that the superhelical turns of covalently closed native PM2 DNA are removed by the addition of IDA at a binding ratio of 0.14 in a solvent containing 0.01 or 0.02 M Na^+. This is significantly lower than the value of 0.17 found in the present work in a solvent containing 0.05 M Na^+. The difference is probably due to the use of a different salt concentration. A significant difference with Waring and Chisholm (1972) is found in the shape of the titration curve in the region of the equivalence point: the asymmetry observed in this region (Figure 12) is not apparent in their results. This asymmetry suggests that the unwinding angle of IDA is not constant, but increases as a function of r. This is consistent with the fact that several effects of IDA on the DNA physicochemical properties are induced cooperatively, especially at salt concentrations above 0.05 M. Since IDA does not intercalate into DNA, its effect on the covalently closed circular PM2 DNA can possibly be due to the opening of a fraction of the DNA base pairs. In effect, Vinograd et al. (1968), Rush and Warner (1970), and Wang (1974) have shown that the superhelical turns of covalently closed circular DNA molecules can be removed by alkaline titration of a fraction of the DNA base pairs, and the initial number of tertiary turns of such molecules has been determined, making the assumption that ten base pairs titrated correspond to the release of one negative superhelical turn. Evidence for the opening by IDA of a fraction of the DNA base pairs is provided by the measurement of the rate of reaction of the bases with formaldehyde: in appropriate conditions, the same order of magnitude of the reaction rate was observed with heat-denatured DNA and with the complex DNA-IDA at a high steroid/phosphate binding ratio; whereas the reaction rate was practically equal to zero with double-stranded DNA. DNA destabilization is observed when more than 0.21 steroid molecule is bound per phosphate residue. From the data of Figure 1, it can be estimated that increasing r by 0.01 causes a decrease of roughly 5 °C of the T_m of the DNA. Fuchs and Daune (1973) observed that the T_m of the DNA in which 1% of the bases are modified by the binding of *N*-2-acetylamino-7-fluorofluorene decreases 1.1 °C. Therefore, it is likely that the number of base pairs opened by one steroid molecule is larger than the number of base pairs opened by one fluorene derivative bound to DNA. Furthermore, the biphasic response of the DNA thermal stability to IDA suggests that in the destabilizing phase of the interaction the number of DNA base pairs open is not a linear function of the amount of bound steroid, in contrast with the stabilizing interaction characterized by a linear increase of T_m as a function of r in the range 0-0.21.

Since the unwinding angle of ethidium is 26° (Wang, 1974; Liu and Wang, 1975), the average unwinding angle of IDA is 7.2° and the actual number of tertiary turns of the closed

DNA molecule extracted from phage PM2 is in our conditions −67. The opening of 7% of the base pairs of this molecule is sufficient to remove these superhelical turns. But the hyperchromicity at 260 nm associated with such a conformational change should be about 3%, while the hyperchromicity actually measured for a steroid binding ratio of 0.17 is 12%. The hyperchromicity induced by IDA and H_2IDA can be due either to a DNA conformational change or to a change of the physical properties of the medium surrounding the DNA and is very likely the result of a combination of these two types of effects. A weak hydrophobic interaction between the steroid nucleus and a specific region of the DNA bihelix is probable. But the free-energy change of the binding reaction is predominantly electrostatic, as shown by the steep decrease of log K as a function of the salt concentration and the stabilizing effect of the steroids against the DNA thermal denaturation (Mahler et al., 1966). Since the highest density of negative charges is found across the bihelix minor groove, it is reasonable to assume that the steroid binds preferentially in that region of the DNA. Supporting this hypothesis is the observation that spermine, which itself binds in the DNA small groove, competes with a high efficiency with IDA for binding to DNA. Model-building studies using space-filling molecular models show that the DNA minor groove provides an adequate binding site for IDA, with its two amino groups coming into close contact with phosphates of the two DNA strands, and the β side of the pregnene nucleus facing the hydrophobic region of the DNA small groove. Malouetine which has a structure closely related to IDA has been shown by NMR to interact with DNA through the methyl groups of the β side of its steroid nucleus (Gourevitch et al., 1974). Molecular model studies suggest that several positions of the steroid within the DNA minor groove are possible, especially at a low binding ratio. When r increases, mutual interaction such as electrostatic and steric repulsion between bound steroids develops and results in a regular arrangement of the molecules in the DNA minor groove. The DNA hyperchromicity results in a direct interaction of the steroids with the DNA chromophores or an indirect interaction through a change of the DNA solvatation, but in any case depends upon the relative position of the steroid and the DNA chromophores. Thus, the assumption that various positions of the steroids in the DNA small groove are possible and that there is a mutual interaction between bound steroids can explain the phenomenom of cooperativity of the hyperchromicity. The different possible positions of the steroids in the DNA small groove can also explain the cooperativity in the competition with ethidium for DNA binding. At a low r value, one IDA molecule prevents the unwinding required for ethidium intercalation of one or two base pairs. At a higher binding ratio and higher r value, two steroids are able to block the unwinding of at least five or six base pairs. Thus, the number of forbidden intercalation sites does not increase linearly as a function of the amount of bound steroids.

The binding free-energy of IDA is higher by 0.4 kcal/mol than the binding free-energy of H_2IDA. This difference which does not change as a function of ionic strength is presumably due to a better fit of the IDA steroid nucleus to the DNA binding-site surface. Finally, the evidence for cross-linking by IDA of the opposite DNA strands is provided by the observation that the DNA denatured in the presence of IDA at a high binding ratio reanneals readily upon raising the salt concentration of the medium.

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