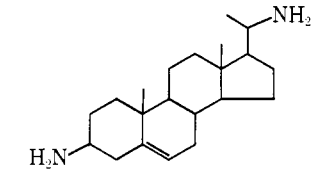
Nucleic Acid–Small Molecule Interactions. VII. Further Characterization of Deoxyribonucleic Acid–Diamino Steroid Complexes*

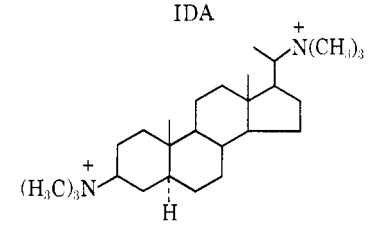
H. R. Mahler,† G. Green, Robert Goutarel,‡ and Q. Khuong-Huu‡

ABSTRACT: The strong, stereospecific interactions, occurring at low ionic strength between deoxyribonucleic acid (DNA) and steroidal diamines such as irehdiamine A (pregn-5-ene-3β,20α-diamine) and malouetine (5α-pregnan-3β,20α-ylenebis(trimethyliodide)) have been investigated by means of five types of optical measurements: absorbance *vs.* temperature profiles, high- and room temperature absorption spectroscopy, as well as optical rotatory dispersion and circular dichroism. The results obtained suggest that in the presence of these ligands two different types of complexes are formed. The first requires a stoichiometry of [steroid]/[DNA-P] $\approx$ 0.20. It is characterized by enhanced thermal stability, increased hyperchromicity at 260 mμ, and a shift of the long-wavelength circular dichroic band toward the red with a considerable increase in rotational strength. It may involve a ligand-induced alteration in the bihelix with a resultant disorientation of the bases away from the B form. The second requires the presence of appropriate diprimary, primary-secondary, or disecondary amines at concentrations of [steroid]/[DNA-P] > 0.3. It is less stable thermally, and at room temperature its optical parameters are those characteristic of partially disoriented DNA. At somewhat higher temperatures this disorientation becomes complete and is retained on recooling. Yet the native degree of order is restored completely on increasing [Na^+] to 0.2 M. This behavior may result from the stacking of steroid micelles, largely on the outside of the helix. The two forms are thought to be related to, but cannot be identical with, those assumed by DNA in the presence of planar, aromatic rings, and Cu^{2+} ions, respectively.

We have previously discussed a number of parameters that appear to govern the interactions of certain steroidal diamines with polynucleotides (Mahler and Dutton, 1964; Mahler *et al.*, 1966). The present report deals with additional experiments designed to establish the specificity and the mechanism of the reaction between DNA, on the one hand, and irehdiamine A[1] (pregn-5-ene-3β,-20α-diamine), its N-methylated derivatives, certain of their isomers, and malouetine (5α-pregnan-3β,20α-ylenebis(trimethyliodide)), on the other. These studies lead us to suggest that complexes of DNA with these molecules can assume two different forms in aqueous solution: one appears to contain a modified helix with a high degree of secondary structure; the other, although devoid of essentially all such structure even at room temperature, re-forms the native bihelix rapidly and completely simply on exposure to high ionic strength.



IDA



Mal

Materials and Methods

Buffers and Sample Preparations. The dialysis buffer is 4.0 $\times$ 10^{-3} M in NaCl, 1.0 $\times$ 10^{-3} M in Tris (Trizma Sigma), and 1.0 $\times$ 10^{-4} M in Na_2-EDTA with the final Ph adjusted to 7.0 at 0°. The working buffer contained 2.4292 g of Tris base and 81.7563 ml of 0.2061 N HCl made up to 100.00 ml (concentration 0.2006 M in Tris; final pH 7.4); when diluted 1:1000 it is 2.006 $\times$ 10^{-4} M

* Contribution No. 1431 from the Chemical Laboratory, Indiana University, Bloomington, Indiana. *Received May 29, 1967.* This research is supported by Grant GM-12228 from the National Institutes of Health.

† Research Career Award PHS GM-05060 of the U. S. Public Health Service.

‡ CNRS Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, Essonne, France.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: IDA, irehdiamine A; Mal, malouetine; h_t, maximal thermal hyperchromicity = [A_{260} (maximal)/A_{260} (15°)] − 1.00; T_m, midpoint of thermal transition, *i.e.*, that temperature in degrees centigrade at which $h = h_t/2$; $\sigma_{s/s}$, dispersion of the transition; r = mole ratio (steroid)/(DNA-P); r_{EP}, r at equivalence point; $s_{20,w}$, sedimentation coefficient corrected to 20° and water; $s^0_{20,w}$, $s_{20,w}$ extrapolated to infinite dilution of macrospecies.

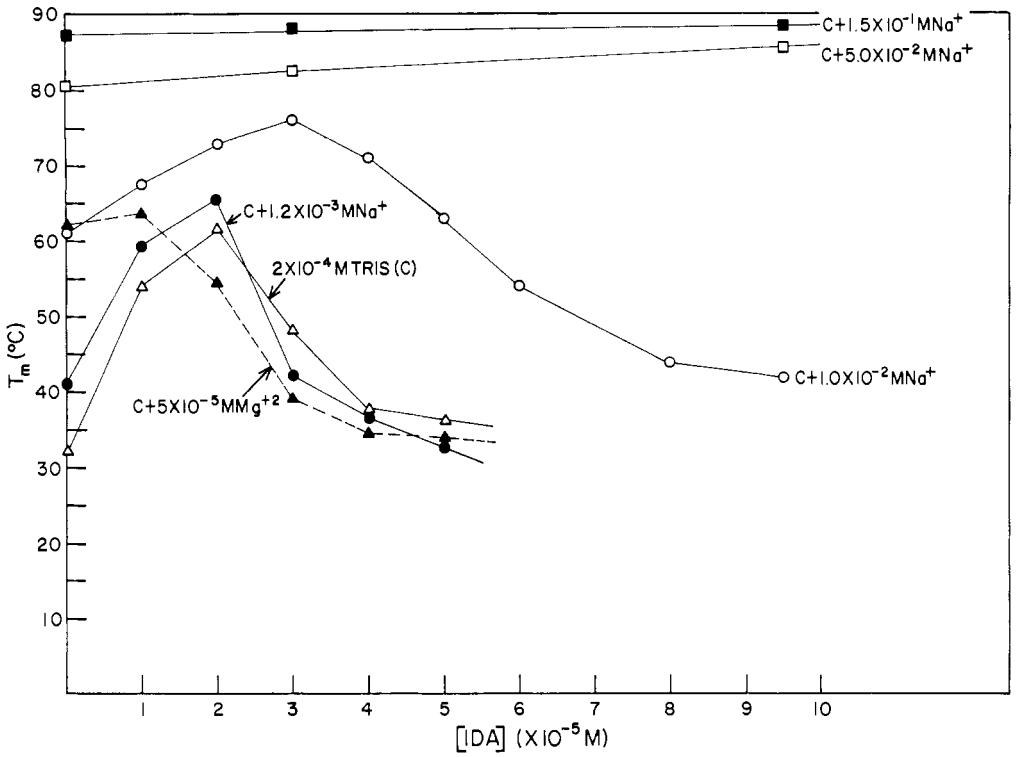


FIGURE 1: Influence of ionic strength on T_m of DNA in presence of IDA^{2+}. *E. coli* DNA (7.5×10^{-5} M DNA-P) in 2.0×10^{-4} M Tris buffer plus IDA at the concentrations indicated was subjected to thermal denaturation, and the T_m obtained from absorbance measurements (curve C). The experiment was repeated in the presence of additional inorganic cations as shown.

Tris buffer; and diluted 1:250 it is 1.0×10^{-3} M Tris. Dialysis tubing (Visking) was pretreated by boiling three times for 5 min alternately in 0.1 M Na_4-EDTA and 0.01 M Tris. DNA solutions were prepared by adding 4.5 mg of sodium DNA (see below) to 4.5 ml of dialysis buffer; after agitation on a wrist action shaker in a cold room at 4°, dissolution was complete after ~30 hr. Stock solutions were prepared by adding sufficient dialysis buffer to yield DNA solutions showing an A_{260} of 0.50. This corresponds to a concentration of DNA-P of 7.5×10^{-5} M since both the calf thymus and *Escherichia coli* DNA used had ϵ_p 6.6×10^3 in dialysis buffer, as well as in 0.20 M [Na^+]. DNA solutions were dialyzed *vs.* six batches of Tris buffer (1.5 l./batch, 6-hr dialysis time for the first five, 12 hr for the last). All other solutions were prepared by adding appropriate stocks of reagents, all in Tris buffer, to the DNA. An equilibration time of 12 hr was allowed before the actual experiment. DNA-P concentrations were calculated on the basis of their absorbance in dialysis buffer measured in a Zeiss Model PMQ spectrophotometer. All glassware was scrupulously acid cleaned and rinsed with glass-distilled water. The latter was used also for the preparation of all solutions.

DNA Samples. Calf thymus DNA was obtained from Worthington Biochemicals, Freehold, N. J., lot no. 995. Its sedimentation and optical properties are described in Mahler *et al.* (1964).

E. coli K-12 DNA was purchased from General Biochemicals, Chagrin Falls, Ohio. Most of the experiments described were performed on several samples of lot no. 650349. This preparation gave an ϵ_p at 260 mμ of 6.60 $\times 10^3$ ($A^{1\%}_{1cm}$ 200), in 0.20 M NaCl–2 $\times 10^{-4}$ M Tris (pH 7.4), and an $s^0_{20,w}$ = 33.55 S in the same solvent. After the dialysis regime indicated above, the T_m of different lots of this preparation was 33 ± 0.5°, $\sigma_{2/3}$ = 8.5 ± 0.5°, h = 0.364. In 1.0×10^{-3} M Tris the T_m was 52 ± 0.5°. On adding 0.2 M NaCl the values were T_m = 88.6°, $\sigma_{2/3}$ = 4.4°, and h = 0.360. Occasionally batches from the same lot gave significantly higher T_m's in Tris buffer, but without any effects on any of the other parameters including the T_m at high ionic strength. This situation could not be remedied by exhaustive dialysis against 0.2 M NaCl, prior to the customary dialysis regime. This change in the base-line value had no effect on ΔT_m in the presence of various ligands, defined as ΔT_m = T_m (DNA plus ligand) − T_m (DNA alone).

Amines. These were prepared at Gif-sur-Yvette. Preparations and properties are described by Janot *et al.* (1962) and Goutarel (1964, and in preparation). Diquaternary amines were obtained as the diiodides, all others as dihydrochlorides. Stock solutions 1.0×10^{-2} or 1.0×10^{-3} M were prepared in Tris buffer and diluted appropriately with the DNA solution to yield the concentrations shown. Usually 1 hr at room temperature was allowed for solutions to reach equilibrium. $IDA(HCl)_2$ was titrated and the two NH^+_3 groups were found to exhibit identical pK_a's of 9.6 ± 0.1. The same result was obtained by back-titration of the free base with acid.

Thermal Denaturation Profiles. A recording thermospectrophotometer (Gilford Instruments) was used as described previously (Mahler *et al.*, 1966). The variables determined were: (a) the hyperchromicity, h =

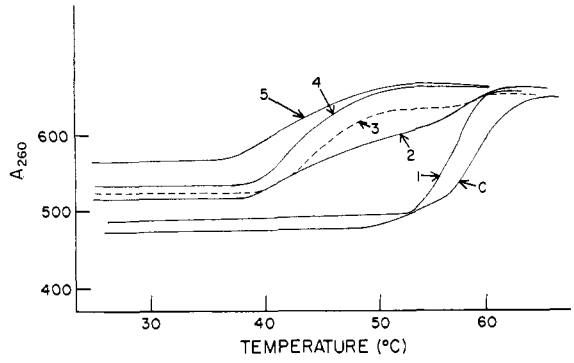


FIGURE 2: Thermospectrograms of DNA in the presence of both Mg^{2+} and IDA^{2+}. Actual trace of absorbance–temperature profiles for the experiments of Figure 1 with [DNA-P] = 7.5×10^{-5} M in the presence of 5.0×10^{-5} M Mg^{2+} are shown. C, control; 1, 1.0×10^{-5} M IDA; 2, 2.0×10^{-5} M IDA; 3, 3.0×10^{-5} M IDA; 4, 4.0×10^{-5} M IDA; and 5, 5.0×10^{-5} M IDA.

A_t/A_{15}, where t and 15 are the experimental and reference temperatures (15°), respectively, and A is the optical density at some wavelength, usually the absorption maximum of the DNA solution; (b) h_t, the maximal thermal hyperchromicity obtained; (c) T_m, the midpoint of the thermal transition, defined as that temperature at which $h = 0.500h_t$; and (d) $\sigma_{2/3}$, the dispersion, defined as that temperature span required to raise h from 0.333 to $0.667h_t$. Absorbance profiles were also monitored at 320 mμ to check for precipitation and aggregation; none was observed.

Optical Measurements. Optical densities were determined either by means of the Gilford thermospectrophotometer calibrated against Gilford standards, or a Zeiss Model PMQII spectrophotometer. Absorption spectra in the ultraviolet region were recorded by means of either a Cary Model 14 spectrophotometer or the Jasco-Durrum recording spectropolarimeter Model ORD–UV-5 operating in the spectrophotometer mode.

Optical rotatory dispersion measurements were recorded by means of the same instrument operating in the spectropolarimeter mode with a high intensity 450-W xenon arc (Osram, Germany) as the light source. The operating temperature was that of the laboratory (27 ± 1°); the polarimeter cells used were of strain-free silica and had a path length between 1.00 and 5.00 cm. All samples were scanned repeatedly against a base line of buffer (or buffer plus ligand) alone in the same cells. For maximal sensitivity with adequate signal-to-noise ratio the instrument was operated at a setting of 30 mdeg or greater for full-scale deflection, and with a scan speed of 4–5 mμ/min. Before recording the instrument was purged with prepurified nitrogen at a flow rate of 10 ft^3/hr. The observed rotations were converted into reduced mean residue rotations $[M']_\lambda$ (Fasman, 1963) by means of the relation $[M']_\lambda = [\alpha]_\lambda \times (mrw/100) \times [3/(n^2+2)]$, where $[\alpha]_\lambda$ is the specific rotation at wavelength λ and equals $\alpha_{obsd}/(lc)$, l is the path length in decimeters, c is the concentration in grams per cubic centimeter, mrw is the mean residue weight (taken as 330), and n is the refractive index of the solvent. The latter was taken to be equal to that of water for the dilute buffer solutions used here (Fasman, 1963). Actually, for the wavelength range employed here, between 230 and 340 mμ, the correction term in brackets equals 0.764 for the lower and 0.784 for the upper limit. Using the mean value 0.774, which is also the correct one at 267.7 mμ, instead *throughout* this range, the maximum error introduced at 230 and 340 mμ equal 1.3 and 1.4%, respectively. This is well below the experimental error and therefore this procedure was adopted in most instances. The values of $[M']$ obtained were independent of concentrations of the nucleic acids used over the range of concentrations employed ($0.800 \leq A_{260} \leq 1.40$), and agreed with those reported by others (Samejima and Yang, 1965), after converting their value of $[\alpha]_{max}$ and $[\alpha]_{min}$ into $[M']$ for the same wavelengths.[2] Originally we used solutions containing KF as counterions as suggested by Samejima and Yang (1964, 1965) but found later that our regular Tris buffer media, even those containing 0.20 M NaCl, were free of significant absorption and rotation over the whole wavelength range, and could thus be used instead.

Circular dichroism measurements were performed with the Durrum-Jasco spectropolarimeter equipped with a circular dichroism attachment. All spectra were run in duplicate, as a check on reproducibility. The Durrum-Jasco instrument provides a direct reading, in absorbance units, of $(A_L - A_R)$ the differential absorption between the right- and left-handed, circularly polarized components. This is then converted into specific ellipticity $[\psi]$ by means of the equation (Sarkar *et al.*, 1967) $[\psi]_\lambda = 33(A_L - A_R)_\lambda/lc$, where A_L and A_R are the measured absorbances of the right- and left-handed circularly polarized light, l is the light path in decimeters, and c is the concentration in grams per milliliter.

The circular dichroic extinction coefficient $\Delta\epsilon_\lambda \equiv (\epsilon_L - \epsilon_R)_\lambda$ is related to $[\psi]_\lambda$, the specific, and $[\theta]_\lambda$, the mean residue ellipticity, by eq 1 (Velluz *et al.*, 1965).

$$\Delta\epsilon_\lambda = \frac{[\theta]_\lambda}{3300} = \frac{[\psi]_\lambda - (mrw/100)}{3300} \approx [\psi] \times 10^{-3} \quad (1)$$

All circular dichroism spectra were run using camphor sulfonic acid as the internal standard. All other operating conditions were the same as for optical rotatory dispersion and are described in the preceding paragraph.

Results

Effect of Ionic Strength on Interactions with IDA. In our previous studies (Mahler and Dutton, 1964; Mahler *et al.*, 1966) we observed that the effect of steroids on the thermal stability of DNA appeared to be strongly influenced by the ionic strength of the medium. We have now investigated this point explicitly by measuring

[2] Their values for *E. coli* DNA at 27° and 0.15 M KF are: λ_{max} and λ_{min} for the extrema of the first Cotton effect equal 290 and 257 mμ with $[M']$ values of 5660 and 5740, respectively. Our values in 0.20 M NaCl–2×10^{-4} M Tris (pH 7.4) at 27° are 292 and 256.5 mμ, with $[M']$ equal to 5650 and 6500, respectively.

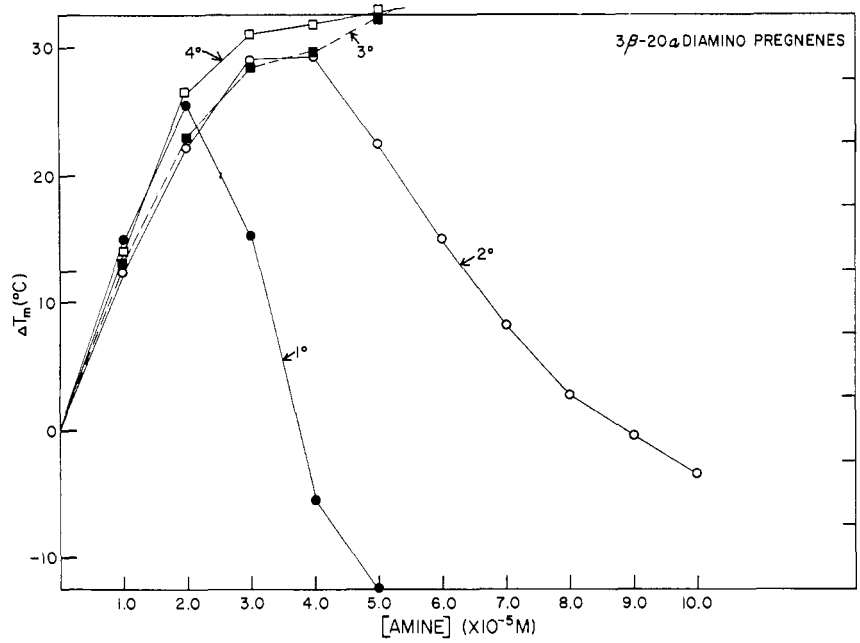


FIGURE 3: Effect of methylation on steroid DNA interactions. *E. coli* DNA (7.5×10^{-5} M DNA-P) in 2.0×10^{-4} M Tris buffer plus steroids at the indicated concentrations was heated denatured and the T_m was obtained from absorbance measurements. $\Delta T_m = T_m$ (with steroid) $- T_m$ (control). All steroids used were 3β,20α-diaminopregnenes bearing the bismethyl substituent indicated primary, none; secondary, monomethyl; tertiary, dimethyl; and quaternary, trimethyl.

the T_m of *E. coli* DNA as a function of IDA concentration in a standard buffer (2.0×10^{-4} M in Tris) in the presence of different concentrations of added NaCl (Figure 1). The concentration of amine required to produce stabilization is virtually unchanged up to and including a $[Na^+] = 5.0 \times 10^{-3}$ M, and similar consideration also applies with regard to the labilizing interaction. Both interactions become less effective (as shown by the change in slope, $d\Delta T_m/d[\text{amine}]$), and the inflection point is shifted to higher concentrations of amine, in 1.0×10^{-2} M salt. The addition of a stoichiometric quantity of Mg^{2+} ions to DNA produces a stabilization equal in magnitude to that produced by 1.0×10^{-2} M Na^{2+}, yet the addition of IDA leads to labilization that appears independent of the presence of the metal ion (compare the slopes of the two curves with and without Mg^{2+} in 2×10^{-4} M Tris). The difference in the effectiveness of Na^+ as compared to Mg^{2+} ions (at concentrations designed to raise the T_m by the same amount) in preventing the labilization by IDA is probably interpretable in terms of greater electrostatic screening by the high concentration of monovalent cation used. At even higher ionic strength ($\geq 5 \times 10^{-2}$ M) no detectable effect attributable to steroidal amines remains. This holds true not only of stabilization to thermal denaturation, but also of all effects on optical parameters (A_{260}, h, optical rotatory dispersion, and circular dichroism) described below, as well as on hydrodynamic parameters such as sedimentation or viscosity.

The effect of ionic strength on the stabilization produced by Mal is quite similar to that observed with IDA. There is an equivalence point, EP, with $r_{EP} = 0.20$ at $[Na^+] \leq 5.0 \times 10^{-3}$ M. At $[Na^+] = 1.0 \times 10^{-2}$ M the position of the equivalence point has not changed but now there is clear evidence of dissociation at the equivalence point. Above a $[Na^+] = 5 \times 10^{-2}$ M, interactions are no longer detectable by any of the techniques used.

We return briefly to the experiments in which IDA is added to Mg–DNA (Figure 2). The results with 1.0, 2.0, and 3.0×10^{-5} M IDA support the notion that the transition is biphasic, with one of the components exhibiting a T_m identical with that of unperturbed Mg–DNA (Olins *et al.*, 1967). Thus, although IDA can re-

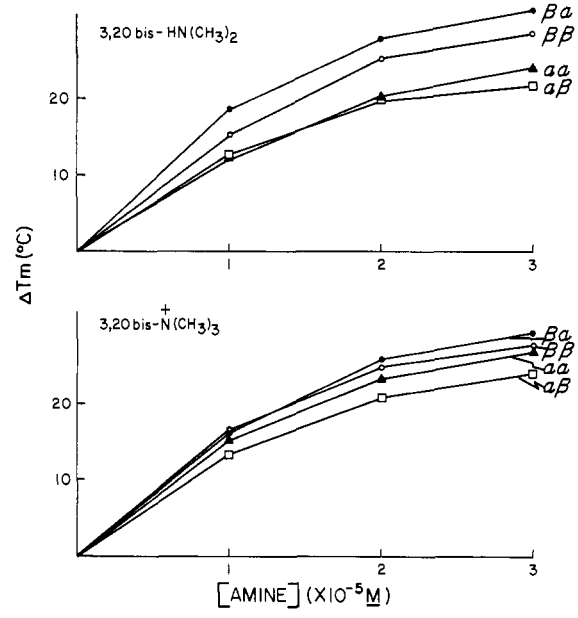
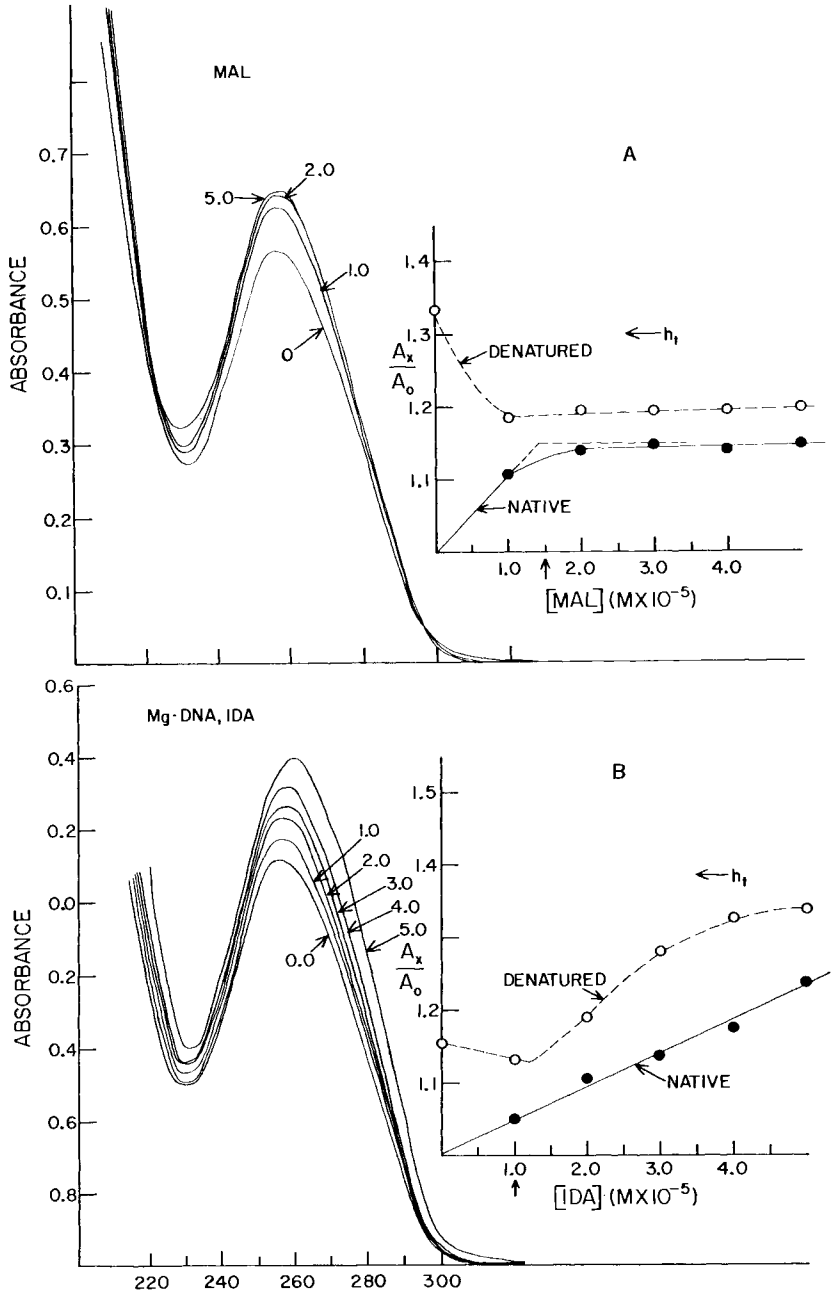


FIGURE 4: Stereospecificity of steroid DNA interactions. *E. coli* DNA (7.5×10^{-5} M DNA-P) in 2.0×10^{-4} M Tris buffer plus steroids was tested as indicated in Figure 3. All steroids used were 3,20-diaminopregnanes with the stereochemistry as shown.



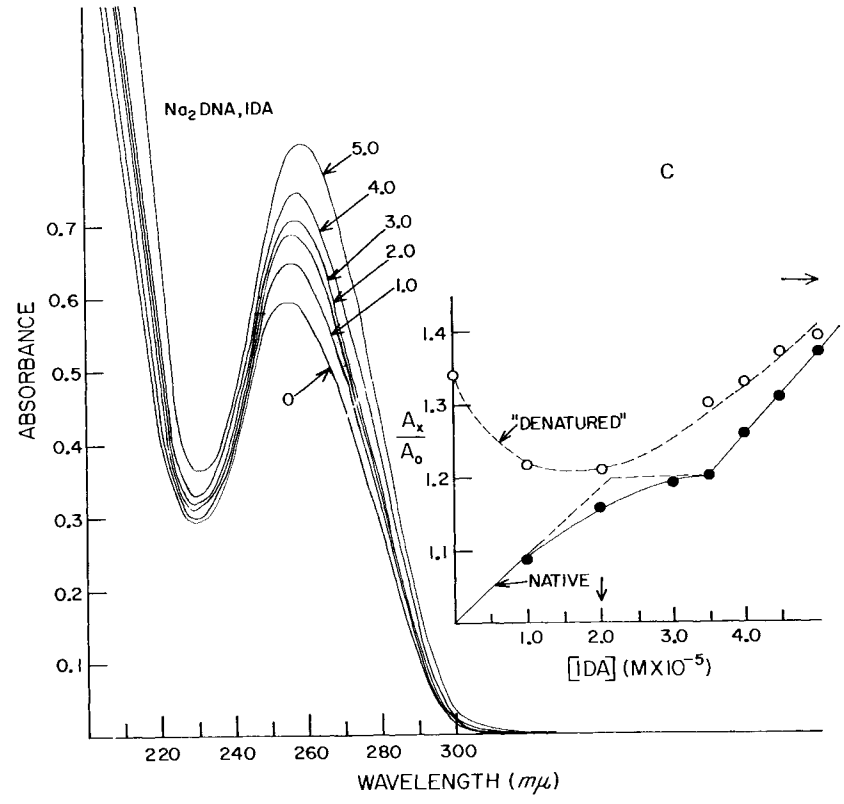


FIGURE 5: Effects of steroids on absorption spectrum of DNA. All experiments with *E. coli* DNA (8×10^{-5} M DNA-P) in 2.0×10^{-4} M Tris buffer. (A) Effects of malouetine: spectra for control (no malouetine added) and malouetine at 1.0, 2.0, and 5.0×10^{-5} M are shown on the left. In the insert are shown the effects of increasing [Mal] on the A_{258} for native and denatured DNA relative to this parameter for native DNA in the absence of ligand. Denatured DNA was produced by heating DNA in 2.0×10^{-4} M Tris buffer to 95°, holding at this temperature for 10 min and quenching in ice. The vertical arrow indicated the position of equivalence point determined from heat denaturation studies; the horizontal arrow indicates the value of $A_{\text{denatured}}/A_{\text{native}}$ for the control obtained from such studies. (B) Effects of IDA on Mg-DNA: spectra for control (DNA in Tris buffer containing 5.0×10^{-5} Mg^{2+}) and IDA at 1.0, 2.0, 3.0, 4.0, and 5.0×10^{-5} M are shown on the left. Data in insert are analoguous to those of part A (C). Effects of IDA on disodium DNA. Spectra for control (DNA in Tris buffer containing 1.0×10^{-3} M Na^+) and IDA at 1.0, 2.0, 3.0, 4.0, and 5.0×10^{-5} M are shown on the left. Data in insert are analogous to those of part A.

place magnesium, magnesium- and IDA-containing molecules or segments seem to melt independently of one another.

Specificity with Regard to Steroid Structure. We have been able to confirm and extend the rules concerning the structural requirements for steroids to be effective (Mahler *et al.*, 1966), due to the availability of three series of steroids: (a) the complete series of bis-*N*-methyl derivatives of IDA, *i.e.*, the stereochemically related diprimary, disecondary, ditertiary, and diquaternary 3β,20α-diaminopregnenes; and, four stereoisomeric 3,20(α,α-, α,β-, β,α-, and β,β-)diaminopregnane derivatives to provide (b), the tertiary bis(dimethylamino), and (c), the quaternary bis(trimethylammonium) series.

The consequences of bismethylation are shown in Figure 3. Stabilizing efficiency appears to fall in the order diquaternary $\geq$ diprimary > ditertiary $\geq$ disecondary. This is approximately the order of ion-pair-forming capacity as measured by the K_b. As reported earlier, labilization is restricted to the diprimary and disecondary derivatives, with the former producing more pronounced effects at lower concentrations.

Certain quantitative differences between different stereoisomers (Figure 4) were also found in both series, the order of effectiveness being $\beta,\alpha > \beta,\beta > \alpha,\alpha > \alpha,\beta$. The configuration 3$\beta$,20$\alpha$ is the one present in the naturally occurring diamines in both the pregnene and the pregnane series.

Optical Studies. EFFECTS ON ABSORBANCE. The hypochroism of the polymer decreases when steroids are added to DNA. In order to assess various contributions to this effect and to establish possible correlations with measurements of thermal denaturation, we have now performed spectrophotometric measurements on both native, and quenched, heat-denatured DNA from *E. coli*: (1) with malouetine, which produces only thermal stabilization; (2) with IDA added to Mg–DNA, *i.e.*, in the presence of Mg^{2+} in an amount stoichiometric to DNA-P, which leads to labilization almost exclusively; and (3) with IDA added to DNA in 1.0×10^{-3} M [Na^+], conditions that lead to the typical biphasic stabilizing and labilizing response (Figures 1 and 3). We have also measured, as an essential control, the ultraviolet absorption spectra of the steroids themselves. They showed no detectable absorbance between 220 and 320 mμ.

The effects of adding amine on the ultraviolet spectrum of native DNA are shown in Figures 5A–C. The inserts represent a replot of the increase in absorbance relative to native DNA (*i.e.*, the hyperchromicity) at the absorption maximum as a function of amine concentration for both native and denatured DNA, and arrows indicate the position of the equivalence point determined by thermal studies. Malouetine is an exclusively stabilizing amine, yet it increases the absorbance and renders native DNA hyperchromic (Figure 5A), presumably by decreasing interactions between transition moments of adjacent bases (DeVoe and Tinoco, 1962a,b).

A similar situation is encountered when IDA is added to the DNA–Mg complex (Figure 5B): except for the first increment of diamine added, the ligand increases the hyperchromicity of this form of DNA. Its absorption maximum shifts from 257 to 265 mμ, and the Cotton effects decrease (see below). Hence the structure of DNA–Mg in the presence of this amine appears to resemble partially melted rather than native DNA, and as we know it also melts out at a lower temperature.

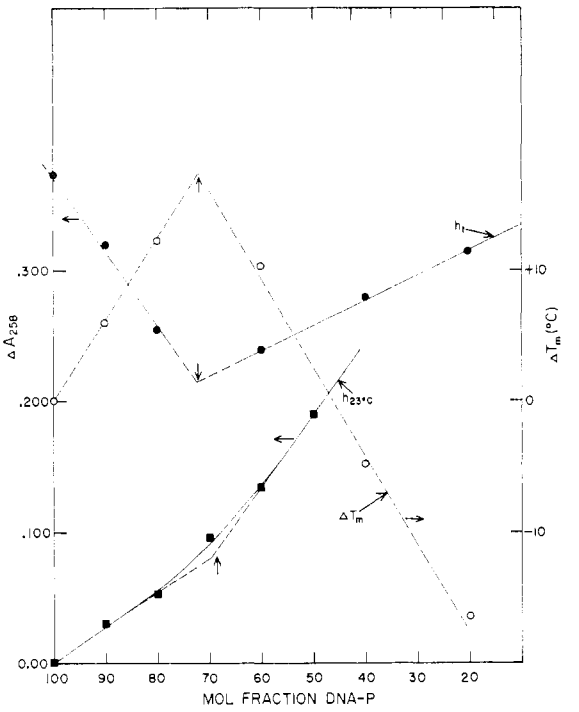


FIGURE 6: Stoichiometry of DNA–IDA interaction by method of continuous variation (Job plot). Solutions of DNA (*E. coli*, DNA-P = 6.75×10^{-5} M) in Tris buffer (1.0×10^{-2} M Na^+) were mixed with solutions of IDA (6.75×10^{-5} M) in the same buffer to yield the mole fractions indicated. Room temperature absorbance ($h_{23°}$), T_m on thermal denaturation, and increase of absorbance after thermal denaturation and heating to 85° (h_t) were recorded. The equivalence points determined from the three measurements are indicated by vertical arrows.

The response of Na_2–DNA to the addition of IDA is biphasic (Figure 5C), quite analogous to its behavior in studies on thermal denaturation. At low concentrations of amine the response is reminiscent of that seen with Mal. At higher concentrations it is that given by the DNA–Mg complex. In fact the optical as well as the thermal data can be accounted for almost completely by simple superposition of the results with Mal and with Mg–DNA: the total concentration dependence on IDA is made up of an initial region closely resembling that observed with Mal, followed by a second one analogous to that produced when IDA is added to Mg–DNA. The values obtained from these determinations (in 2×10^{-2} M Tris, Figure 5A), as well as from a Job (1928) plot of hypochromicity *vs.* mole fraction (values for 0.01 M Na^+ in 2×10^{-4} M Tris shown in Figure 6) all lead to satisfactory agreement regarding the stoichiometry of complex formation between DNA and IDA with $r_{EP} \approx 0.3$. Completely analogous observations also obtain in 1.0×10^{-3} M Tris; here ΔT_m at the equivalence point = 26°, yet r_{EP} still equals 0.3. For denatured (heated and quenched) DNA the effect of adding

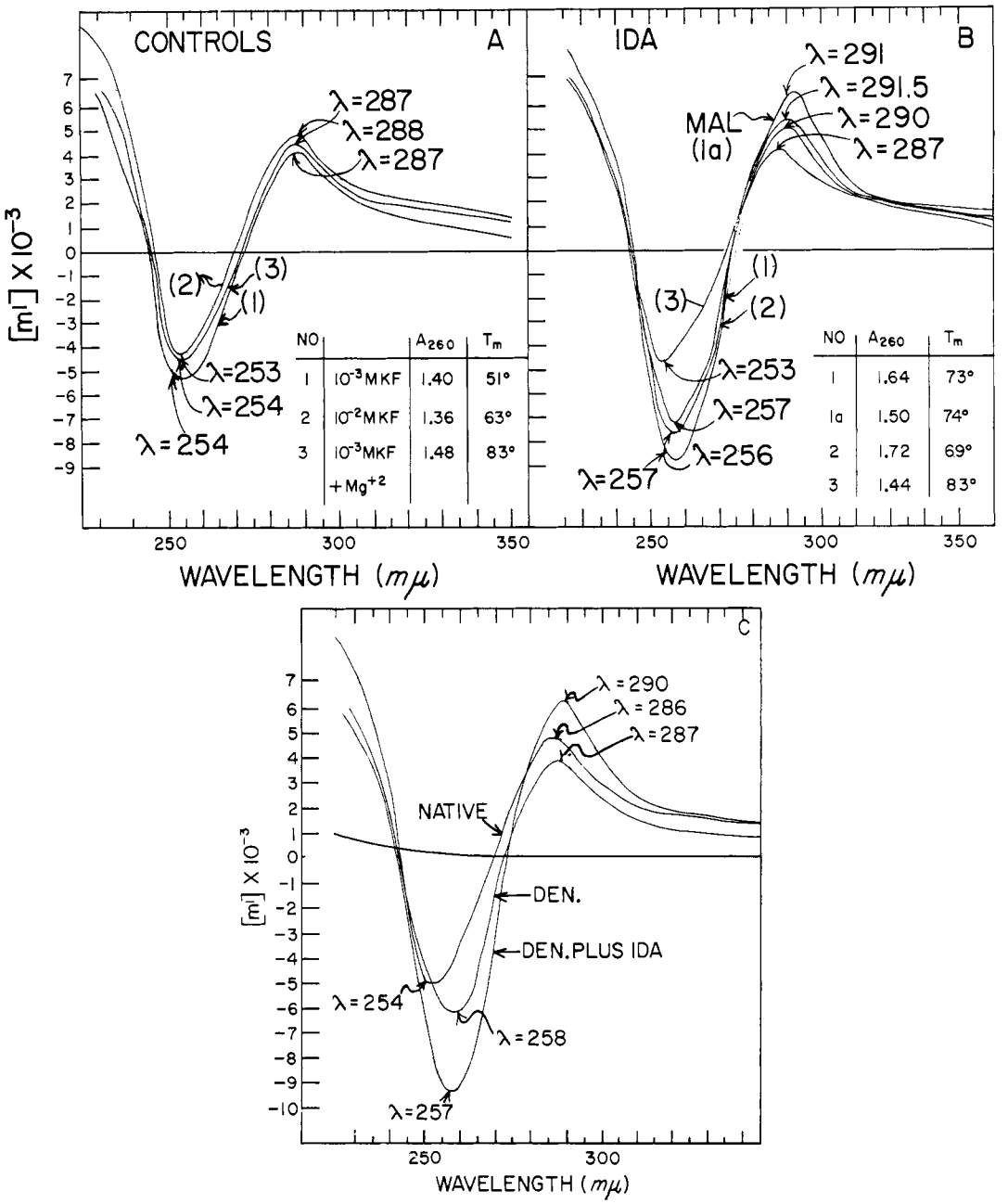


FIGURE 7: Effects of steroids on optical rotatory dispersion spectra of DNA. DNA used was calf thymus DNA with absorbance as shown in the media indicated. (A) Native DNA controls. (B) Curves 1-3, native DNA in the media of part A with 5.0×10^{-5} M IDA; curve 1a similar to 1 but with 5.0×10^{-5} M malouetine. (C) Denatured DNA: DNA in medium 1 (part A) heated to 95°, held for 10 min, and quenched. Curves shown are for native DNA, denatured DNA, and denatured DNA in the presence of 5.0×10^{-5} M IDA.

either amine is opposite that exhibited by the native form. The absorbance changes indicate complex formation, but now the complex is hypochromic relative to the uncomplexed form (Figure 5A,C, insert). The apparent r_{EP} for this interaction is of a magnitude similar to that for native DNA.

EFFECTS ON OPTICAL ROTARY DISPERSION AND CIRCULAR DICHROISM. The use of optical rotatory dispersion and circular dichroism measurements for the study of secondary structure in nucleic acids and other polynucleotides is becoming ever more pervasive (see, for instance, Samejima and Yang, 1964, 1965; Fasman *et al.*, 1964, 1965; Brahms and Mommaerts, 1964; Brahms, 1965; Vournakis and Scheraga, 1966; Ts'o *et al.*, 1966; Gratzer, 1966; Bush and Tinoco, 1967). The power of the method is such as to furnish information not only with respect to the extent of order and secondary structure in, say DNA and its alteration in the course of various disordering processes, but also to permit, at least a start toward, assessing the quantitative contributions made by the various components of such secondary structures. These are principally of two kinds: the vertical stability provided by base-stacking forces (dipole–dipole interactions, etc.) and the horizontal one provided by base–

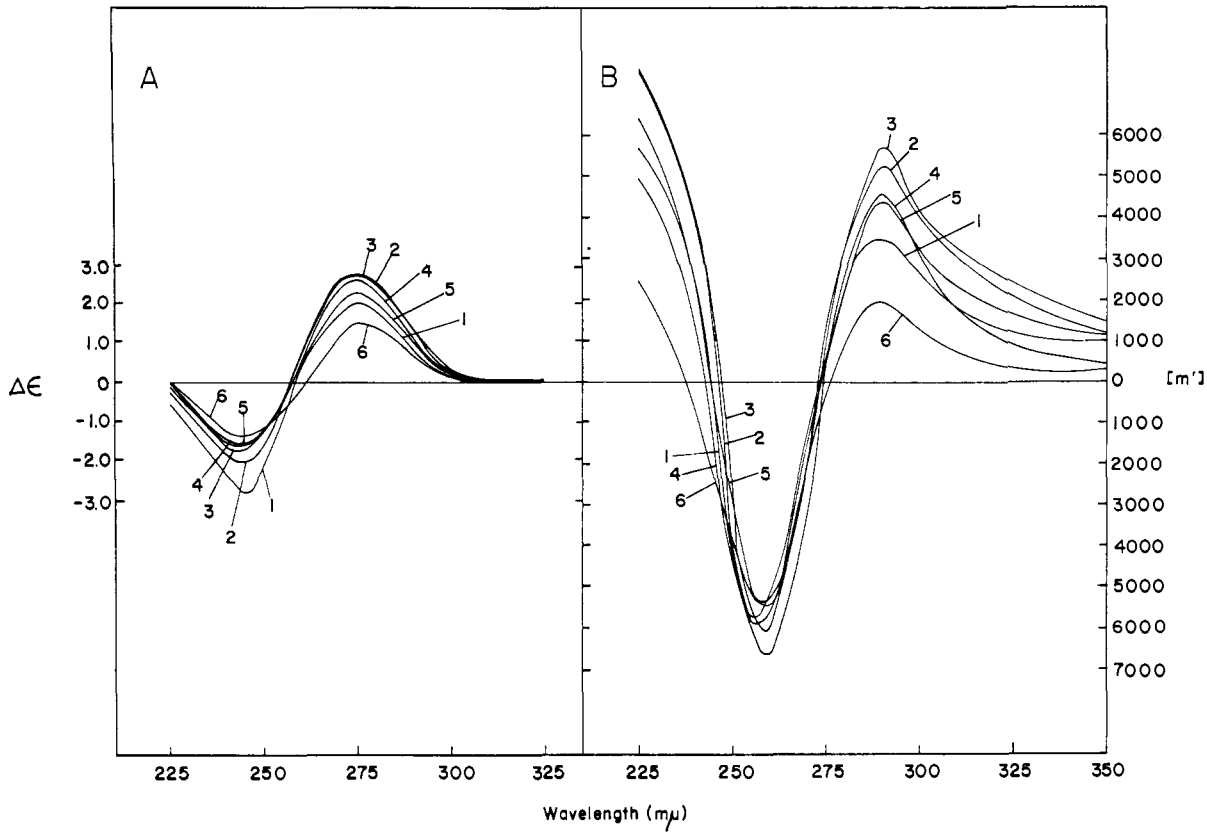


FIGURE 8: Titration of *E. coli* DNA with IDA. (A) Circular dichroism and (B) optical rotatory dispersions. To *E. coli* DNA (DNA-P = 1.18×10^{-4} M) in 1.0×10^{-3} M Tris (pH 7.4) (curve 1) were added increments of IDA to final concentrations of 1.0×10^{-5} M (curve 2), 2.0×10^{-5} M (curve 3), 3.3×10^{-5} M (curve 4), 5.0×10^{-5} M (curve 5), and 1.0×10^{-4} M (curve 6). Aliquots were removed; one was used without dilution for determination of circular dichroism and optical rotatory dispersion spectra (all in the Jasco-Durrum instrument, see *Materials and Methods*); the other was diluted with an equal volume of buffer and used for room temperature-absorbance measurements and thermal denaturation studies (Table I).

base hydrogen bonding (*cf.* references just cited plus DeVoe and Tinoco, 1962a; Sinanoglu and Abdulnur, 1964; Bautz and Bautz, 1964; Crothers *et al.*, 1965; Holcomb and Tinoco, 1965; Cantor and Tinoco, 1965; Warshaw and Tinoco, 1965; Cantor *et al.*, 1966; Vournakis *et al.*, 1966; Michelson and Monny, 1966). It thus appeared appropriate to extend our investigations to the determination of the optical rotatory dispersion spectra of native and denatured DNA in the presence of Mg^{2+}, IDA, and Mal, all at concentrations producing approximately equal thermal stabilization for native DNA. The optical rotatory dispersion and circular dichroism spectra of the amines at 10^{-4} M do not differ from the base line between 225 and 350 mμ. Typical optical rotatory dispersion spectra for calf thymus DNA are presented as Figures 6A,B and 7 for the native and denatured forms, respectively.

Steroid ligands generate increased Cotton effects with denatured DNA. This indicates an increase in rotational strength and is referrable to an alteration of higher order structure, at least over relatively short regions. With native DNA, steroids also produce an enhancement of the Cotton effect, in contrast to Mg^{2+} which somewhat decreased the effect (Cheng, 1965). This last observation appears surprising at first glance since the addition of the metal ion profoundly enhances the thermal stability of the polymer (Figure 1). One must remember, however, that optical rotatory dispersion is responsive not only to the extent but also the kind of higher order structure in a polymer, and it is not yet always immediately obvious how to dissect out the relative contributions of these two sets of parameters. Thus, for instance, the mean residue rotation of RNA of various types is actually greater than that in DNA, in spite of the greater extent of hydrogen bonding and helicity commonly ascribed to the latter (Samejima and Yang, 1964, 1965; Brahms, 1965; Michelson *et al.*, 1966; Vournakis and Scheraga, 1966; Ts'o *et al.*, 1966; Gratzer, 1966). We also note the following. (a) The positions of the extrema for the Cotton effect of disodium DNA and Mg–DNA are virtually superimposable. Similarly the mean residue rotation of disodium DNA over the whole spectrum appears insensitive to variations in the ionic strength of the counter ion between the limits investigated (from 1.0×10^{-3} to 2×10^{-2} M). For Mg–DNA there is, as already mentioned, only a slight decrease in magnitude. Since it is likely that the maximum number of hydrogen bonds are probably present in all these forms, there may exist subtle differences in the stacking of the bases in the Mg–DNA molecule compared to that in DNA with Na^+ as counter ion. The ϵ_P of Mg–DNA is 6.30×10^3 while that for disodium DNA

TABLE 1: Correlation of Thermal and Optical Measurements for IDA–DNA Interactions.[a]

Sample	r [IDA]/[DNA-P]	$\Delta\epsilon_{260}$	h_t	ΔT_m (°C)	$\Delta[M']_{290}$	$\Delta[\theta]_{275}$
1	0.000		1.26			
2	0.085	0.160	1.22	6.9	860	2640
3	0.170	0.500	1.18	16.8	1520	2810
4	0.280	0.580	1.14	26.1	2180	2310
5	0.423	0.775	1.14	16.9	1090	980
6	0.850	0.900	1.05	−3.6	−1360	−1980

[a] All optical rotatory dispersion, circular dichroism, and thermal measurements were performed in 1.0×10^{-3} M Tris (pH 7.4) as described under Materials and Methods. $\Delta\epsilon_{260}$ is the room temperature hyperchromic extinction at 260 mμ. $\Delta[M']_{290}$ and $\Delta[\theta]_{275}$ are the increases in mean residue rotation (definitions given under Materials and Methods) and ellipticity at the positive maxima, 290 and 275 mμ, respectively (see Figure 8). T_m of the control sample was 53.1°; $\epsilon_{260} = 6.60 \times 10^3$, $[M']_{290} = 3330$, and $[\theta]_{275} = 6600$.

is 6.60×10^3 at 260 mμ. (b) All these optical parameters are qualitatively different with steroidal diamines as ligands. The optical rotatory dispersion is red shifted and the mean residue rotations of the positive extremum are increased, as are the ϵ_P (to 7.15×10^3 for IDA, and 7.25×10^3 for Mal at an $r = 0.2$).

A complete titration of *E. coli* DNA in 1.0×10^{-3} M Tris (pH 7.4) with IDA by means of optical rotatory dispersion and circular dichroism is presented in Figure 8. Circular dichroism is capable of providing data complementary to but easier to interpret than those of optical rotatory dispersion (Moscowitz, 1960; Beychok, 1966; Van Holde *et al.*, 1965). In agreement with data already in the literature (Brahms and Mommaerts, 1964; Sarkar *et al.*, 1967) and expected on theoretical grounds (Tinoco, 1964) the main absorption band in DNA (λ_{max} 260 mμ) generates two dichroic bands, centered at that wavelength (λ_0), of almost equal magnitude but of opposite sign (here at λ_{max} 275 mμ, λ_{min} 245 mμ). The interaction with IDA results in parallel effects on the long-wavelength extremum of the optical rotatory dispersion spectrum (cross-over at 275 mμ) and on the positive dichroic band. In both optical rotatory dispersion and circular dichroism we observe an enchancement at low [IDA]/[DNA] ratios with a slight shift of λ_0 and λ_{max}, followed by a decrease at higher ratios, with a final value considerably below that for disodium DNA (Table I). The value of r at which the enhancement reaches its maximum value lies between 0.2 and 0.3, in agreement with the observations on room temperature ultraviolet absorption, and thermal denaturation, described previously (Table I). The effect of IDA on the two components of the dichroism is, however, unequal; while the positive band increases (*e.g.*, $[\theta]_{275}$ is enhanced by 38% at $r = 0.170$) the negative one actually decreases ($[\theta]_{245}$ diminishes by 27% at the same ratio).

Reversibility. TERMINOLOGY. In discussing the possible reversibility of conformational changes of DNA in solution we will adhere to the terminology recently suggested by Kohn *et al.* (1966). In this terminology native DNA is ordinary bihelical DNA; melted DNA is the form produced by disorientation processes such as heat, etc., under conditions when the bihelix is not thermodynamically stable; this melted DNA is frequently, but not always, a random coil conformation, and melting may, but need not necessarily, lead to strand separation (*i.e.*, no strand separation in reversible, *i.e.*, cross-linked DNA); denatured DNA refers to a conformation not possessing long-range helical order, but existing under conditions in which double-helical DNA is thermodynamically stable (it is ordinarily produced from melted DNA by a reversal of the melting process). The process that converts denatured into native DNA is called renaturation while the one that reverses melting is called zippering-up. In addition we need a term describing the stabilized form of DNA present with added IDA at low concentrations or Mal throughout its concentration range. We shall call this form stabilized DNA, and on melting refer to it as melted, stabilized DNA. Finally we also require a term for the complex of DNA with higher concentrations of IDA, and destabilized relative to its uncomplexed form. We propose to call it labile DNA and after melting, melted, labile DNA.

TWO-STEP EXPERIMENTS (TABLE II). In the first series of experiments, native, stabilized, and labile DNA, all from *E. coli*, were subjected to an ordinary thermal melting experiment. These runs were performed in 2.0×10^{-4} M Tris, but similar results have also been obtained in other media varying in ionic strength from 2.0×10^{-4} to 1.0×10^{-2} M. In all instances the r required to produce stabilized DNA was taken at approximately 0.5 r_{EP}, and the one for labile DNA was taken at ~2.0 $\times$ r_{EP} (Figure 1). In order to see to what extent the melted forms were subject to either zippering-up or renaturation on recooling, samples were withdrawn, both at a temperature before the transition had actually been completed and at one >20° above that at which the absorption had become virtually constant, and cooled back down to room temperature. There was no zippering-up of the control, of stabilized, or of labile DNA on cooling and reheating, provided these samples had been raised to a temperature well above their T_m. These re-

TABLE II: Heat Denaturation of *E. coli* DNA.

Expt	Conditions	a_0[a]	a_t[a]	h (a_t/a_0 − 1)	T_m (°C)	$\sigma_{2/3}$ (°C)
1	a. Control (6.4×10^{-5} M DNA-P) in 2×10^{-4} M Tris	1.000	1.364	0.364	42.6	11.9
	b. a, heat denatured (65°), quenched, or slow cooled	1.300	1.375	0.075	40	15
	c. a, heat denatured, quenched, or slow cooled, plus NaCl to 0.20 M	1.150	1.295[b]	0.145	<45[b]	35
9	a. 6.4×10^{-5} M DNA in 2×10^{-4} M Tris–0.20 M NaCl	0.900	1.220	0.360	88.0	4.4
	b. a, with 4×10^{-5} M IDA added	0.880	1.220	0.380	88.6	4.7
12	a. IDA at 4.0×10^{-5} M ($r = 0.625$) in 2×10^{-4} M Tris (DNA-P = 6.4×10^{-5} M)	1.220	1.500	0.226	36.1	12.7
	b. a, heat denatured (80°), quenched, or slow cooled	1.495	1.505			
	c. a, heat denatured, quenched, added NaCl to 0.20 M	0.970[c]	1.320	0.360	86.8	4.6
23	DNA (6.4×10^{-5} M DNA-P) plus IDA at 1.0×10^{-5} M in Tris buffer heated to 80°, added IDA to 4.0×10^{-5} M, slow cooled, added NaCl to 0.20 M	1.120	1.340	0.190	43	17
24	Similar to 12c, but dialyzed *vs.* 2.0×10^{-4} M Tris prior to addition of salt	0.890[d]	1.130	0.273	88.5	7.0
25	Similar to 24, but dialysis preceeded final heating	0.920	1.150	0.250	88.2	4.6
43	c. Similar to 1c, but with DNA-P[e] 1.0×10^{-4} M; IDA at $r = 0.650$	0.995	1.290	0.320	89.3	5.0
	b. a, heat denatured (80°) quenched	1.320	1.42			
	c. Add NaCl to 0.20 M	0.970	1.243	0.280	90.5	6.1
50	c. Similar to 1c[f] but DNA-P = 1.17×10^{-4} M, $r = 0.557$	1.00	1.195	0.195	87.7	5.5

[a] Relative to absorbance in 2.10^{-4} M Tris at room temperature. [b] Approximately 10% of the total hyperchromicity observed was contributed by a component with a $T_m \geq 85°$. [c] 1.050 on addition of salt, dropped to the value shown between 22 and 45°. [d] 0.942 on addition of salt, dropped to the value shown between 22 and 45°. [e] New batch of DNA; T_m in 0.20 M NaCl is 90.3°, $\sigma_{2/3} = 4.3°$. [f] third batch; T_m in 0.20 M NaCl = 89.0°; $\sigma_{2/3} = 5.0°$.

sults were unaffected whether cooling was fast or slow (at the rate of 1°/min). If samples were removed at an earlier part of the heating cycle, then both control and stabilized DNA appeared to be capable of limited zippering-up, although the bulk of the molecules (or parts of molecules) exhibiting this second-step transition was certainly accounted for by that portion not yet melted out during the first heating. In any event, addition of IDA to melted, stabilized DNA to make its concentration sufficient to produce labile DNA, in no way enhanced this effect. Labile DNA behaved quite differently: both samples, even the one removed before the completion of the first transition, not only appeared to be completely denatured (*i.e.*, the melted form did not zipper-up), but this melting (in the sense of removal of secondary structure) appeared to be much more extensive than with either of the other two forms. In fact, as judged by its hyperchromicity, there was *no* return of even short-range order in the conversion of labile melted into denatured DNA. Unlike all other cases investigated and reported in the literature, in this instance denatured DNA at room temperature appeared to be as deficient in structure as its melted conformation at temperatures well above the T_m.

Even more startling was the behavior of this type of DNA on exposure to high ionic strength. If a sample exposed to the first heating or even one subject to the two successive heating steps just described was quenched in ice, returned to room temperature, and made 0.2 M in NaCl, it exhibited a virtually quantitative capacity to form bihelical, native DNA. Its room temperature absorbance dropped, virtually instanta-

neously to that of a native sample and its melting behavior, without any indication of extensive prior annealing, was indistinguishable from that of appropriate controls. This behavior is unique to melted, labile DNA; melted control, or melted, stabilized DNA showed no indication of this capability.

We are faced with a triple paradox. (1) Relatively high concentrations of IDA ($r = >0.3$) are required. These concentrations, before melting, produce labile DNA, a low-melting, less structured conformation. Lower concentrations, *i.e.*, those producing the more highly structured, stabilized DNA are ineffective. Ligand at these higher concentrations must be present during melting. If it is added subsequently, it is ineffective. (2) Complete restoration of bihelical structure by addition of salt at low temperatures has taken place starting with a highly disoriented structure, but the presence of IDA during this restructuring is probably not required (expt 24 and 9). (3) It takes place under conditions where the denatured DNA has retained the structure of melted DNA (see next section). The process therefore constitutes renaturation rather than just a zippering-up, yet it does not appear to be constrained by a kinetic barrier and require prolonged annealing at a temperature below T_m, as is true of most renaturing processes (Marmur *et al.*, 1963). In order to explore this paradox further we have performed some additional experiments.

TYPE I *vs.* TYPE II DENATURATION. Geiduschek (1962) has shown how, by conducting thermal transition measurements both at ambient (d assay) and, after reequilibration, at room temperature (i assay), one can distinguish melting from denaturation, and zippering-up from renaturation (type I *vs.* type II reversibility). We have performed such experiments and some results are shown in Figure 9A–C.[3] Figure 9A shows the native DNA control (7.5×10^{-5} DNA-P in 2×10^{-4} M Tris). It is analogous to many of those described by Geiduschek; the two curves are not superimposable, and the difference between the two midpoints equals $\sim 3°$, a reasonable result for this low ionic strength. Figure 9B describes the analogous experiment for stabilized DNA (1.1×10^{-5} M IDA, $r = 0.15$). Stabilization, *i.e.*, an increase in T_m is observed for both, the d as well as the i assay, but the latter is relatively inaccurate because of the greatly reduced hyperchromicity. Figure 9C describes the experiments with labile DNA (*i.e.*, in the presence of 4.5×10^{-5} M IDA, $r = 0.60$). We detect a wholly novel behavior; d assay and i assay melting curves coincide. This is opposite of what is observed with cross-linked DNA and indicates, at least by the reasoning used in this field so far, that the two strands must have become completely unwound.

ALKALINE DENATURATION. We have tried to determine whether the effects of IDA during denaturation by alkali are similar to those just described for thermal denaturation. To this end we have exposed *E. coli* DNA in 1.0×10^{-3} M Tris to a pH of 12.1 (0.01 M KOH) for 30 min. During this treatment A_{260} of the control increased from 0.450 to 0.520 while the IDA-treated sample ($r = 0.5$) rose from 0.515 to 0.545; after reneutralization (0.01 M HCl) the values remained elevated at 0.575 and 0.565, respectively. Correspondingly the peak–trough amplitude in the optical rotatory dispersion dropped from 9200 to 7700 in the control, and from 11,200 to 7500 in the IDA-treated sample; after reneutralization these values remained virtually unchanged. After raising the salt concentration to 0.2 M NaCl, however, all parameters instantly became identical with those characteristic of disodium DNA at that high ionic strength and the resulting DNA solution melted sharply at 87.1° ($h = 0.37$). Therefore, although exposure to high pH at this ionic strength has certainly denatured the DNA, the strands have not become separated even after 30 min and this entanglement leads to instant zippering-up on exposure to high ionic strength. This stands in marked contrast to the behavior of DNA at high pH and high ionic strength (≥ 0.2 M Na^+); under these conditions the unwinding time is of the order of seconds (Freese and Freese, 1963; Davison, 1966).

PHYSICAL PROPERTIES. In order to extend and confirm the observations based on thermal denaturation profiles, we also investigated the optical rotatory and circular dichroic behavior of native, distorted, and labile DNA after melting, followed by exposure to high ionic strength. The optical rotatory dispersion results are shown in Figure 10A–C. Labile DNA, as already described in Figure 8, shows a shift toward longer wavelengths, and also exhibits the diminution in rotational strength characteristic of partially disordered polynucleotides (Samejima and Yang, 1965; Ts'o *et al.*, 1966). On melting and quenching (denaturation), a similar effect now becomes apparent in the control; there is an increase in rotational strength of the stabilized DNA and a more dramatic decrease in the labile DNA. On exposure to concentrated electrolyte all three samples exhibit virtually identical optical rotatory dispersion spectra. Thus optical rotatory dispersion, in this instance, cannot be considered a really specific indicator of the re-formation of bihelical structure. Identical results were also obtained in 1.0×10^{-3} M Tris, where in addition, they were confirmed by means of circular dichroism. Here the ellipticity after heat treatment of labile DNA showed an additional decrement of 30% and returned to the control value after addition of salt.

Mal only produces stabilized DNA at all concentrations tested, as measured by any of the methods just described, and mole for mole is somewhat more efficient than is IDA in this range.

We have also investigated the hydrodynamic parameters $s^0_{20,w}$ and η on addition of diamines, melting, and increase in ionic strength. The specific viscosity, at least, behaves in the expected fashion, exhibiting a drop in all samples, followed by an approximate return to the control value for labile DNA only. But we have not as yet determined the concentration dependence of this

[3] We have also run a second cycle d assay on all the reequilibrated (*i* assay) samples. In all instances the profiles observed corresponded exactly to those of the first cycle d assay except for the variation in initial absorbance; *e.g.*, one sample was removed at the temperature of the vertical line, at an A_{260} of 0.580 it was cooled and reequilibrated and now gave an A_{260} of 0.545. On reheating it coincided with the first heating curve (d assay) from an initial A_{260} of 0.545.

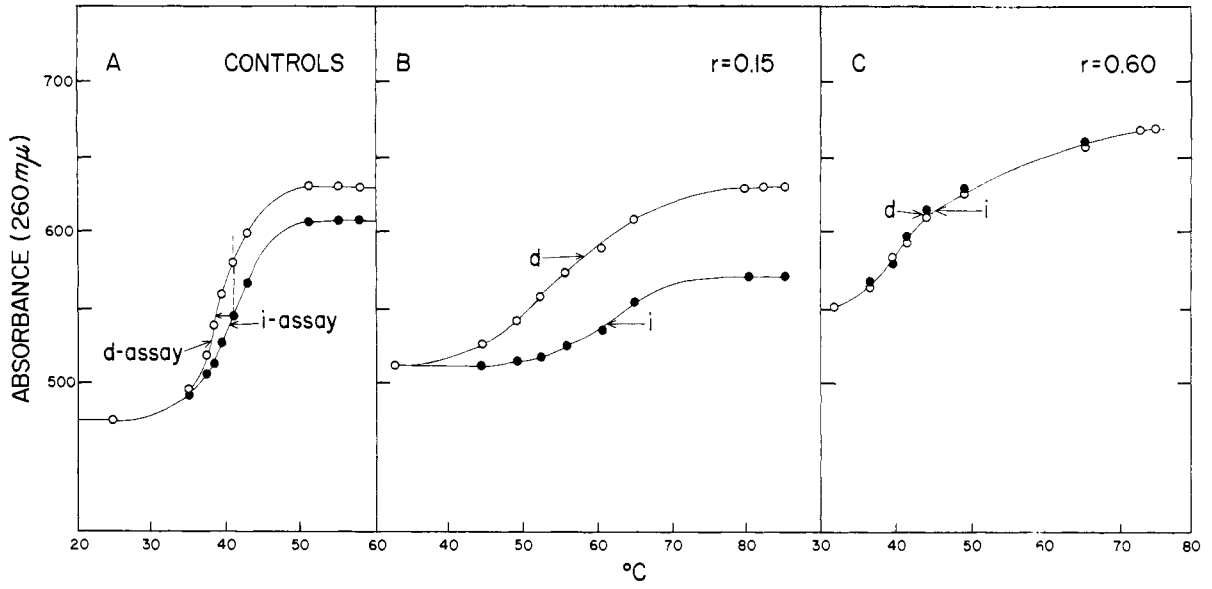


FIGURE 9: Thermal denaturation by d and i assay. *E. coli* DNA (DNA-P at 7.5×10^{-5} M) in Tris buffer. Effect of temperature on absorbance at λ_{max} measured either at ambient temperature (d assay) or after cooling, and equilibration at 23° for 24 hr (i assay). (A) Control, (B) with IDA at $r = 0.15$, and (C) with IDA at $r = 0.60$.

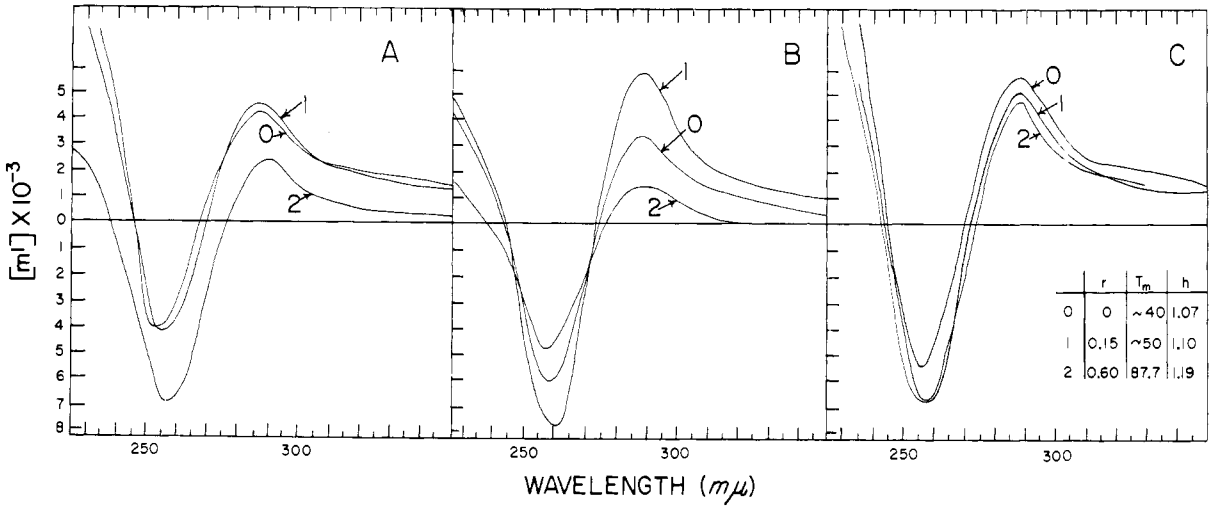


FIGURE 10: Effect of IDA on optical rotatory dispersion spectra of *E. coli* DNA. DNA (0.1 mg/ml) was dialyzed extensively *vs.* Tris buffer and made up to a concentration of 1.3×10^{-4} M DNA-P; the control (curve O) contained no IDA; curves 1 and 2 correspond to solutions containing 1.5 and 6.5×10^{-5} M IDA, respectively. (A) Native DNA. (B) DNA after denaturation at $(T_m + 20)°$ and recooling to room temperature. (C) As in B, but after adding NaCl to a final concentration of 0.20 M. Thermal data are shown in insert. h is here $A_{max}/A_{23°}$.

effect and therefore can make no statement concerning $[\eta]$. For $s_{20,w}$, where we have done so, present indications are that the series of treatments necessary to produce the results described lead to some degradation during melting of distorted, and to aggregation of labile DNA, after the addition of salt. In a representative experiment with the preparation of *E. coli* DNA described the values for $s^0_{20,w}$ were: for the unheated control 33.5, and for the heated control 34.6; for heated, stabilized DNA 18, and for heated, labile DNA[4] 384, 284, 208, and 170 all in 0.20 M NaCl. The formation of aggregates or networks after renaturation is by no means uncommon (see for instance Waring and Britten, 1966).

Interpretation and Conclusions

The experiments reported here provide additional information concerning two questions raised in previous studies of this series: (a) the existence of two types of complexes between steroidal diamines and DNA; (b) possible structures of these complexes, especially with respect to their macromolecular component.

As concerns point a we now see that (1) all diamines tested are capable of strongly interacting with native DNA; (2) the stoichiometry of this interaction, origi-

[4] Uncorrected sedimentation coefficients for 0.025 mg/ml.

nally inferred from thermal melting experiments, can also be measured by means of optical parameters (A_{260}, optical rotatory dispersion, and circular dichroism); the results obtained are in agreement and suggest a mole ratio of [amine]/[DNA-P] $\simeq$ 1:4; (3) even though 1 holds, both its qualitative (*i.e.*, whether the interaction produces stabilization or labilization) and quantitative aspects depend on the structure of the steroid used and give evidence of structural and even stereochemical specificity.

As concerns point b it may be well to summarize the properties established so far for each of the two types of complexes. Complex I is formed when steroidal diamines at a relatively low concentration are added to DNA. It is formed with a stoichiometry of approximately 4 [DNA-P]/[diamine] (ionic strength ≤ 0.01), the stoichiometry is relatively insensitive to amine structure. The complex appears more stable than disodium DNA; it melts with a T_m some 20° greater than that observed for disodium DNA, but with much less cooperativeness. Its extinction coefficient is greater than that of disodium DNA, *i.e.*, it is hyperchromic with respect to the latter. Its optical properties are as follows. In optical rotatory dispersion there is an enhancement and a slight red shift of the positive extremum with virtually no alteration in cross-over or negative extremum, relative to uncomplexed DNA. In circular dichroism the rotational strength of the positive dichroic band centered at 275 mμ is enhanced some 50% without any effect on the position of its maximum, while that of the negative band is somewhat decreased with an apparent blue shift. In assessing the possible significance of these observations we must first establish the nature of the electronic transitions responsible for the optical rotatory dispersion and circular dichroism effects observed. Pertinent facts are that (a) IDA exhibits no chromophore at any wavelength >225 mμ; (b) neither IDA nor malouetine exhibit any optical activity in the absence of DNA over the whole wavelength range from 225 to 350 mμ; (c) dihydro-IDA lacks even the C=C chromophore of the parent compound, yet behaves exactly like IDA in all respects in its interaction with DNA, including optical rotatory dispersion and circular dichroism spectra (Mahler *et al.*, 1965; G. Green, unpublished observations); (d) the effects on optical rotatory dispersion and especially circular dichroism spectra are concerned with a band (most readily followed at the long-wavelength extremum of the optical rotatory dispersion curve, or the positive circular dichroism band) which previous work has assigned to a conformation-sensitive electronic transition in nucleic acids (*i.e.*, the main π–π^* transition). This set of observations makes it likely that what is affected is DNA structure by virtue of a perturbation due to diamine and not *vice versa*. Of particular interest is the combination of decrease in hypochromicity (*i.e.*, an increase in oscillator strength) with an increase in the dichroism (*i.e.*, in rotational strength). Since the integrated absorption coefficient is proportional to the square of the total electric dipole moment for an electronic transition while the rotational strength is the scalar product of the electric and magnetic dipole moments for the same transition, this combination strongly suggests the possibility of a structural (configurational of conformational) change in the macromolecule (Moscovitz, 1960; Lin *et al.*, 1964). The question then arises whether this alteration is conformational (*i.e.*, concerned with secondary structure) rather than configurational (*i.e.*, concerned with primary structure). Since presumably no new covalent bonds are formed in the complex, any effect, including one on hydrogen bonds is, *prima facie*, conformational. The enhancement of thermal stability also points in that direction. What sort of conformational change can produce all the effects observed? One has to search for one that combines enhanced rotational strength with decreased interactions between transition moments of nearest neighbors, and one that exhibits a decrease in cooperativity of melting as well. A more extended helix, analoguous to that assumed by DNA in the presence of low concentrations of intercalated acridines or other planar, polynuclear aromatic dyes (Lerman, 1961, 1963, 1964; Waring, 1966a,b; Kersten *et al.*, 1966), would be consistent with the observations presented. Indeed mutations induced in coliphage T2 by proflavine are reverted by IDA (Mahler and Baylor, 1967). Yet if there is such a change in conformation of DNA it cannot be brought about by intercalation *per se*, for steroids cannot intercalate; models show that their rings are puckered, not planar, and the bulky bistertiary or quaternary groups in some of the most effective ones (*e.g.*, malouetine) make substitution for a base pair impossible. Another feasible alternative might be one in which the propinquity of the steroid has forced the bases in the macromolecule into a form analogous to that assumed in the A form of DNA (Franklin and Gosling, 1953; Langridge *et al.*, 1957, 1960), where the bases are tilted and are no longer perpendicular to the helix axis. Conformations of this type have been suggested by Ts'o *et al.* (1966) for polyribonucleotides, compounds which differ from their polydeoxy analogs precisely by showing enhanced rotational strength, and by Luzzati *et al.* (1964) for the structure of DNA in pure ethylene glycol. Some tentative support for this hypothesis is provided by the observation that addition of IDA in stabilizing concentration renders the circular dichroism curve more RNA-like, not only by increasing rotational strength at 275 mμ, but also by increasing the absence of symmetry between the positive and negative lobes of the circular dichroism; in RNA the latter is virtually absent (Brahms and Mommaerts, 1964; Sarkar *et al.*, 1967). Whatever the details, the thermodynamic driving force for putting the DNA into its altered structure is provided by the negative free energy of complexation with IDA, a complex that probably entails "chelation" of two phosphate residues on DNA electrostatically with the diammonium form of the steroid.

Complex II is formed at relatively high ($r \geq 0.5$) concentrations of diprimary or disecondary amines. The complex is thermally unstable; under appropriate conditions it melts with a T_m even lower than disodium DNA. It is hyperchromic with respect to the latter, and optical rotatory dispersion as well as circular dichroism indicate extensive destruction of order, analogous to other forms of partially melted DNA. Yet even after complete elimination of all remaining secondary struc-

ture by heating, as measured by any of the optical criteria (absorption, optical rotatory dispersion, and circular dichroism) the native, bihelical structure can apparently be restored completely by simply raising the ionic strength.

At first glance this particular combination of properties appears highly bizarre. Still, the interactions of Cu^{2+} ions with DNA described by Eichhorn (Eichhorn and Clark, 1965; Eichhorn *et al.*, 1966) and by Hiai (1965) bear many remarkable resemblances to those just reported. Both types of agents produce a lowering of T_m; both generate hyperchromicity in denatured DNA; both lead to a thorough disorientation and hyperchromicity on melting of native DNA which cannot be reversed on cooling, yet is instantly and extensively reversible by exposure to high ionic strength; both are ineffective in forming complexes with DNA at high ionic strength. Yet there are important differences also. The concentration of Cu^{2+} required is relatively high, and even at 5×10^{-3} M Na^+, T_m titrations of 5×10^{-5} M P equivalents of DNA require $\geq 1.0 \times 10^{-4}$ M Cu^{2+}; the effects of Cu^{2+} are reported to be independent of the G–C content of the DNA in contrast to what we observe with the steroids (Mahler *et al.*, 1966); the effect of Cu^{2+} is always labilizing, never stabilizing, in contrast to the biphasic response to certain of the steroids; steroids in the labilizing range produce partial hyperchromicity not only in denatured but also in native DNA; the thermal transitions in the presence of Cu^{2+} are hypersharp, while those in the presence of steroids are broad; and finally, and most importantly, Cu^{2+} is a relatively small ion (even as a hydrate), capable of penetrating the helix and reacting with the nucleic acid bases, and such chelation is known to occur with isolated bases, nucleosides, or nucleotides. Corresponding reactions with steroids are not known, nor particularly likely. Thus the explanation advanced to account for the Cu^{2+} effect in terms of metal-mediated, interbase cross-links is probably not tenable in the present instance. Instead we would like to suggest that the formation of steroid micelles on the outside of the helix, proposed previously (Mahler et al., 1966), may well lead to extensive disorientation. In spite of this almost complete loss of order it may equally well interfere with, rather than facilitate, complete strand separation. The latter necessarily requires that the complementary strands unwind (Freese and Freese, 1963; Crothers, 1964; Davison, 1966), and this rotation may be effectively blocked by the greater viscous drag of the adhering steroid micelles. If some of these aggregates are brought into the interior of the helix during such "abortive" unwinding, then neither renaturation to the native bihelical form nor rezippering to labile DNA can take place on cooling to room temperature. The former is, in any event, not favored thermodynamically in the presence of high concentrations of steroid; the latter is physically and kinetically blocked by steroid molecules which now occupy spaces not only outside but inside the original volume of the helix as well. Yet the bases themselves may have retained their proper orientation, and therefore, immediately on removal of the blocking steroid by exposure to salt, the extended but interlaced double coils with their bases still in register become thermodynamically unstable with respect to bihelical DNA, and may rapidly and extensively revert to the latter. The situation may be quite analoguous to that reported here for DNA denatured by alkali at low ionic strength, followed by reneutralization and exposure to high ionic strength.

The effects of steroids on DNA described here and in previous publications are complex and not yet susceptible to interpretation in terms of any simple model. We hope that studies on simple homopolynucleotides, currently in progress, will serve to fill this gap.

Acknowledgment

The authors are indebted to Professor M. M. Janot, Director of the Institut des Substances Naturelles, for his support and encouragement. We also thank Mr. Grant Gansinger and Miss Celia Weber for their careful experimental work.

References

Bautz, E. K. F., and Bautz, F. A. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1476.
Beychok, S. (1966), *Science 154*, 1288.
Brahms, J. (1965), *J. Mol. Biol. 11*, 785.
Brahms, J., and Mommaerts, W. F. H. M. (1964), *J. Mol. Biol. 10*, 73.
Bush, C. A., and Tinoco, I., Jr. (1967), *J. Mol. Biol. 23*, 601.
Cantor, C. R., Jaskunis, S. R., and Tinoco, I., Jr. (1966), *J. Mol. Biol. 20*, 39.
Cantor, C. R., and Tinoco, I., Jr. (1965), *J. Mol. Biol. 13*, 65.
Cheng, P.-Y. (1965), *Biochim. Biophys. Acta. 102*, 314.
Crothers, D. (1964), *J. Mol. Biol. 9*, 712.
Crothers, D., Kallenbach, N., and Zimm, B. H. (1965), *J. Mol. Biol. 11*, 802.
Davison, P. F. (1966), *J. Mol. Biol. 22*, 97.
DeVoe, H., and Tinoco, I., Jr. (1962a), *J. Mol. Biol. 4*, 500.
DeVoe, H., and Tinoco, I., Jr. (1962b), *J. Mol. Biol. 4*, 518.
Eichhorn, G. L., and Clark, P. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 586.
Eichhorn, G. L., Clark, P., and Becher, E. Z. (1966), *Biochemistry 5*, 245.
Fasman, G. D. (1963), *Methods Enzymol. 6*, 928.
Fasman, G. D., Lindblow, C., and Grossman, L. (1964), *Biochemistry 3*, 1015.
Fasman, G. D., Lindblow, C., and Seaman, E. (1965), *J. Mol. Biol. 12*, 630.
Franklin, R. E., and Gosling, R. G. (1953), *Acta Cryst. 6*, 673.
Freese, E. B., and Freese, E. (1963), *Biochemistry 2*, 707.
Geiduschek, E. P. (1962), *J. Mol. Biol. 4*, 476.
Goutarel, R. (1964), Alcaloïdes Steroïdiques des Apocynacees, Paris, Hermann.

Gratzer, W. B. (1966), *Biochim. Biophys. Acta 123*, 431.
Hiai, S. (1965), *J. Mol. Biol. 11*, 672.
Holcomb, D. N., and Tinoco, I., Jr. (1965), *Biopolymers 3*, 121.
Janot, M. M., Laine, F., Khuong-Huu, Q., and Goutarel, R. (1962), *Bull. Soc. Chim.*, 111.
Job, J. (1928), *Ann. Chim. 9*, 113.
Kersten, W., Kersten, H., and Szybalski, W. (1966), *Biochemistry 5*, 236.
Kohn, K. W., Spears, C. L., and Doty, P. (1966), *J. Mol. Biol. 19*, 266.
Langridge, R., Seeds, W. E., Wilson, H. R., Hooper, C. W., Wilkins, M. H. F., and Hamilton, L. D. (1957), *J. Biophys. Biochem. Cytol. 3*, 767.
Langridge, R., Wilson, H. R., Hooper, C. W., Wilkins, M. H. F., and Hamilton, L. D. (1960), *J. Mol. Biol. 2*, 19.
Lerman, L. S. (1961), *J. Mol. Biol. 3*, 18.
Lerman, L. S. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 94.
Lerman, L. S. (1964), *J. Cellular Comp. Physiol. 64*, 1.
Lin, C. Y., Urry, D. W., and Eyring, H. (1964), *Biochem. Biophys. Res. Commun. 17*, 642.
Luzzati, V., Mathis, A., Masson, F., and Witz, J. (1964), *J. Mol. Biol. 10*, 28.
Mahler, H. R., and Baylor, M. B. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 256.
Mahler, H. R., and Dutton, G. (1964), *J. Mol. Biol. 10*, 157.
Mahler, H. R., Goutarel, R., Khuong-Huu, Q., and Ho, M. T. (1966), *Biochemistry 5*, 2177.
Mahler, H. R., Kline, B., and Mehrotra, B. D. (1964), *J. Mol. Biol. 9*, 801.
Marmur, J., Rownd, R., and Schildkraut, C. L. (1963), *Progr. Nucleic Acid Res. 1*, 231.
Michelson, A. M., and Monny, C. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1528.
Michelson, A. M., Ulbricht, T. L. V., Emerson, T. R., and Swan, R. J. (1966), *Nature 209*, 873.
Moscowitz, A. (1960), *in* Optical Rotatory Dispersion, Djerassi, C., Ed., New York, N. Y., McMillan, Chapter 12.
Olins, D., Olins, A., and von Hippel, P. (1967), *J. Mol. Biol. 24*, 151.
Sarkar, P. K., Wells, B., and Yang, J. T. (1967), *J. Mol. Biol. 25*, 563.
Sarkar, P. K., and Yang, J. T. (1965), *J. Biol. Chem. 240*, 2088.
Samejima, T., and Yang, J. T. (1964), *Biochemistry 3*, 613.
Samejima, T., and Yang, J. T. (1965), *J. Biol. Chem. 240*, 2094.
Sinanoglu, O., and Abdulnur, S. (1964), *J. Photochem. Photobiol. 3*, 333.
Sinanoglu, O., and Abdulnur, S. (1965), *Federation Proc. 24*, 5.
Tinoco, I., Jr. (1964), *J. Am. Chem. Soc. 86*, 297.
Ts'o, P. O. P., Rapaport, S. A., and Bollum, F. J. (1966), *Biochemistry 5*, 4153.
Van Holde, K. K., Brahms, J., and Michelson, A. M. (1965), *J. Mol. Biol. 12*, 726.
Velluz, L., Legrand, M., and Grosjean, M. (1965), Optical Circular Dichroism, New York, N. Y., Academic.
Vournakis, J. N., and Scheraga, H. A. (1966), *Biochemistry 5*, 2997.
Vournakis, J. N., Scheraga, H. A., Rushizky, G. W., and Sober, H. A. (1966), *Biopolymers 4*, 33.
Waring, M. J. (1966a), *Symp. Soc. Gen. Microbiol. 16*, 235.
Waring, M. J. (1966b), *Biochim. Biophys. Acta 114*, 234.
Waring, M. J., and Britten, R. (1966), *Science 154*, 791.
Warshaw, M. W., and Tinoco, I., Jr. (1965), *J. Mol. Biol. 13*, 54.