atoms, and possibly also the C–O–C oxygen atoms of the deoxyribose ring.

The absorption in the 1550–1720 cm.$^{-1}$ region in the spectrum of deuterated DNA is due to C=O and ring stretching vibrations of the purine and pyrimidine bases. The additional band observed at 1690–1712 cm.$^{-1}$ in the non-deuterated spectrum has a contribution from the bending vibration of the NH and NH_2 groups. Since changes in these bands have just begun to occur at 65% r.h., we conclude that sites "4" and "5" do not begin to hydrate until near 65% r.h.

Conclusions.—The hydration behavior of DNA may be summarized: between 0 and 60 to 65% r.h. water adsorbs on sites provided by the $PO_2^-Na^+$ portions of the DNA backbone. The P–O–C and C–O–C oxygens probably also become hydrated below 65% r.h. At 65% r.h. about 6 water molecules are adsorbed completing the hydration of the phosphate group.[19] The C=O groups and ring nitrogen atoms become hydrated above 65% r.h. A transition from a low r.h. structure to the high r.h. helical form of DNA occurs between about 60 and 75% r.h.[11,20] Above 75% the water molecules hydrating the C=O groups and ring nitrogen atoms fill the grooves of the DNA helix. This process is complete by about 80% r.h. and further hydration of DNA is accompanied by swelling.[12] All exposed hydration sites are probably filled at this point.

(19) It is significant that this value corresponds closely to the hydration shell of about 6.5 water molecules per nucleotide for DNA in aqueous solution found by J. H. Wang, *J. Am. Chem. Soc.*, **77**, 258 (1955).

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[CONTRIBUTION FROM THE SPECTROSCOPY LABORATORY AND THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY, CAMBRIDGE, MASSACHUSETTS]

Hydration of Deoxyribonucleic Acid. III. A Spectroscopic Study of the Effect of Hydration on the Structure of Deoxyribonucleic Acid[1]

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The polarized infrared and ultraviolet spectra of oriented films of the sodium salt of deoxyribonucleic acid (DNA) have been investigated as a function of relative humidity (r.h.). Quantitative data are presented for the behavior of the dichroic ratios of the infrared band at 1660 cm.$^{-1}$ and the ultraviolet band at 2600 Å. and for the absorbance of the latter. The curves obtained are congruent, showing one plateau above 75% r.h. and a second below 55% r.h. Between 75 and 55% r.h. there occurs a sharp increase in the dichroic ratios and absorbance at 2600 Å. These changes are reversible. The increase in ultraviolet absorption is similar to that observed when DNA is denatured in aqueous solution by thermal or other means. It is concluded that DNA films are stable in the B configuration at r.h. values as low as 75% and that at still lower humidities a reversible transition occurs to a disordered form in which the bases are no longer stacked one above another and are no longer perpendicular to the axis of the helix. The loss of base stacking upon drying suggests that the B configuration of DNA is stabilized by the stacking of the bases in the presence of water. The B configuration may thus be considered a kind of micelle structure (as postulated by Herskovits, Singer and Geiduschek), in which the number of attractive water–water and base–base interactions is maximized.

Introduction

Numerous studies of the structure of the sodium and lithium salts of deoxyribonucleic acid (DNA) in the solid state have been made using the techniques of X-ray diffraction and several distinct structures have been found to exist in various relative humidity (r.h.) regions.[3–8] Studies using polarized infrared radiation have been made but in these either r.h. was not varied[9] or the investigators were mainly interested in providing infrared evidence for the B⇄A and B⇄C transitions and did not study in detail the behavior of spectra at lower r.h.[10,11]

The ultraviolet spectrum of DNA does not appear to have been studied as a function of r.h.

In this paper are presented the results of a study of the changes occurring in the polarized infrared and ultraviolet spectra of DNA films and of the changes occurring in the absorbance of the band at 2600 Å. as a function of r.h. Such data allow inferences to be drawn concerning structural changes occurring as a function of hydration and provide information about the sources of stabilization energy of the helical configuration of DNA.

Experimental

Infrared.—Apart from the additional techniques described below, samples, hygrostatic cells, and procedures used to obtain polarization spectra were the same as in the preceeding paper.[12] Data are presented only for the sodium salt of deoxyribonucleic acid (NaDNA). Most polarization spectra were recorded on the Perkin–Elmer Model 137 spectrophotometer equipped with a mechanical guide which assured the correct repositioning of the hygrostatic cell on successive runs. Two types of polarizers were used: a wire grid polarizer in the 4000 to 1400 cm.$^{-1}$ region,[13] and a seven-sheet silver chloride polarizer below 1400 cm.$^{-1}$. These were mounted just before the exit slit of the instrument and thus polarized the radiation in both beams. Since each polarizer is essentially 100% effective, the moderate polarizing property of the instrument does not affect the measured dichroic ratio when the polarizer is placed in this position. Spectra were run with the electric vector of the radiation first parallel and then perpendicular to the slits by rotating the polarizer 90°.

Ultraviolet.—The spectra were recorded on a Beckman DK-2 spectrophotometer using a dichroic polarizer generously provided by Mr. A. Makas of the Polaroid Corporation. This polarizer was tested and found to be nearly a perfect polarizer at 2700 Å. and therefore dichroic measurements were made at this wave length. The polarizer was placed at the exit slit and was therefore in both beams. The entire sample chamber of the Beckman instrument was humidified by employing saturated salt solutions.[14]

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(2) Atlantic Regional Laboratory, National Research Council (Canada), Halifax, Canada.

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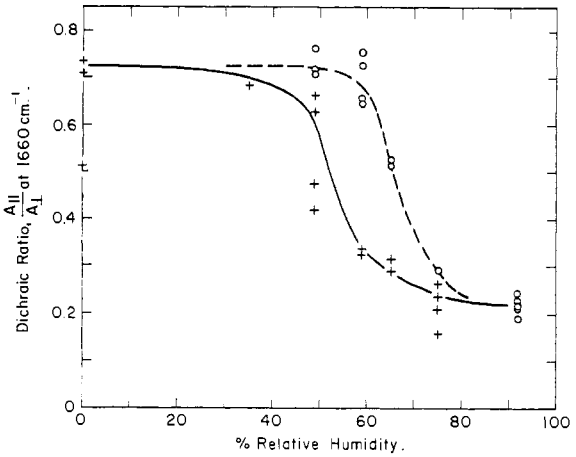

Fig. 1.—The dichroic ratio of the band at 1660 cm.$^{-1}$ in deuterated NaDNA as a function of relative humidity. Circles and crosses indicate values reached by increasing and decreasing r.h., respectively.

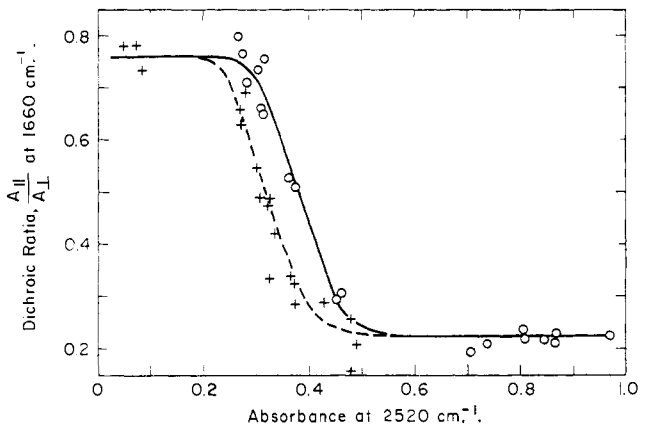

Fig. 2.—The dichroic ratio of the 1660 cm.$^{-1}$ band in deuterated NaDNA as a function of the absorbance of the D_2O band at 2500 cm.$^{-1}$. Circles and crosses as in Fig. 1.

Sample Preparation.—Oriented DNA films were prepared by stroking viscous solutions on a suitable plate with a spatula. Plates of silver chloride were used for infrared work and fluorite disks for the ultraviolet measurements. The film thicknesses employed were, respectively, 1 to 5 microns and about 0.5 micron. To obtain data, the scale of r.h. values was traversed in both ascending and descending directions and equilibration times of days, and sometimes even weeks, were required to assure the constancy of the absorbance values and of the dichroic ratios. The dichroic ratio (R) is $A_{\parallel}/A_{\perp}$, where $A_{\parallel}$ and $A_{\perp}$ are the absorbances of a given band with the E-vector of the plane-polarized radiation respectively parallel and perpendicular to the direction in which the film was stroked. At 1660 cm.$^{-1}$ in the infrared R was reproducible to ±10% while in the ultraviolet R at 2700 Å. was reproducible to ±3% and A at 2700 Å. to ±1%.

Results

Infrared Spectrum.—The spectrum of oriented NaDNA films recorded using polarized radiation changes as the r.h. is varied. At 92% r.h. the bands arising from carbonyl and ring vibrations of the purine and pyrimidine bases which occur in the range 1720 to 1550 cm.$^{-1}$ show perpendicular dichroism, as do bands arising from aromatic C–H stretching at 3103 cm.$^{-1}$, and the bands in the 1150 to 950 cm.$^{-1}$ region.[15] Those at 890 and 775 cm.$^{-1}$ show parallel dichroism. The band at 1240–1220 cm.$^{-1}$ which arises from the antisymmetric PO_2^- stretching vibration exhibits maximum absorbance at different frequencies for the perpendicular and for the parallel components of the band.[15] As r.h. is lowered below 75%, the dichroism of all bands rapidly decreases until at 49% nearly all dichroism has disappeared. Little change occurs between 49 and 0% r.h.

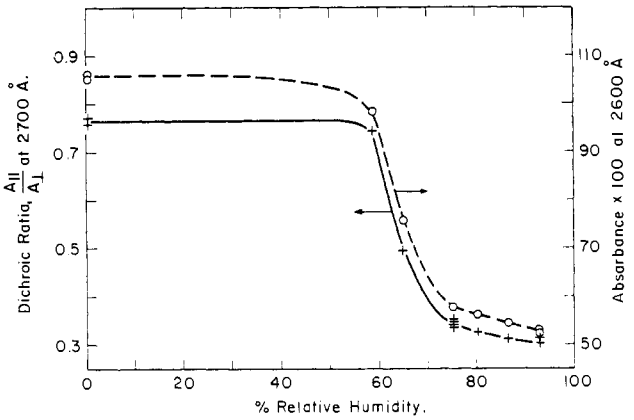

Fig. 3.—The dichroic ratio at 2700 Å. (crosses) and the absorbance at 2600 Å. (circles) as a function of r.h. Data were obtained by increasing r.h.

Figure 1 shows a plot of the dichroic ratio measured at 1660 cm.$^{-1}$ against r.h. for a deuterated film of NaDNA. This curve may be described as having two plateaus, one above 75% r.h. and one below 55% r.h. A region of sharp transition accompanied by hysteresis is observed between 75 and 55% r.h. Figure 2 shows the behavior of the dichroic ratio when plotted against the absorbance of the band at 2520 cm.$^{-1}$ due to adsorbed D_2O. This method of presentation eliminates that part of the hysteresis inherent in the hydration process.[12,14] Thus Fig. 2 shows additional hysteresis between the state of hydration and the structure. These results were reproduced for five films of NaDNA. The behavior of other dichroic bands was similar.

Ultraviolet Spectrum.—The absorbance at 2600 Å. in the spectrum of a representative film of NaDNA is plotted as a function of r.h. in Fig. 3. The absorbance value at 93% r.h. is 0.53 and changes slightly to 0.58 at 75% r.h., then increases sharply to 0.99 at 50% r.h., reaching a final value of about 1.06 at 0% r.h. The value of the dichroic ratio at 2700 Å. is also plotted as a function of r.h. in Fig. 3. The dichroic ratio at 93% r.h. is 0.31 and increases slightly to 0.34 at 75% r.h., then increases sharply to 0.74 at 59% r.h., reaching a final value of about 0.77 at 0% r.h. Hysteresis was observed for both absorbance and dichroic ratio in the 50 to 75% r.h. region. Similar results were obtained with three other oriented NaDNA films. The curve of absorbance *vs.* r.h. also was reproduced with unoriented films. The two curves in Fig. 3 are identical and are very similar to the infrared results presented in Fig. 1.

Both the infrared and the ultraviolet spectral changes were found to be reversible, except for hysteresis effects noted above, and were observed over many cycles of hydration and dehydration of the DNA films.

Discussion

Infrared and Ultraviolet Dichroism.—X-Ray diffraction studies of fibers have shown that NaDNA exists in at least three distinct structural forms in the solid state. The B helical configuration is known to exist above 85% r.h.[5] and is a helical duplex in which the planar purine–pyrimidine base-pairs are stacked perpendicular to the axis of the helix. Between 80% and 75% r.h. the A helical configuration exists.[5] In the transition from the B to the A form, a systematic tilting of the base-pairs occurs such that the angle between the helix axis and the plane containing a base-pair (de-

(14) M. Falk, K. A. Hartman, Jr., and R. C. Lord, *J. Am. Chem. Soc.*, **84**, 3843 (1962).

(15) The above results are in general agreement with previous workers.[9-11] See ref. 12 for a discussion of assignments.

fined here as θ) is equal to 70°. A change of pitch also occurs and is accompanied by a 25 to 30% decrease in length of the DNA fibers.[3] As r.h. is lowered from 75 to 55% r.h., a "disordered state" is obtained which gives diffraction pictures showing no structure.[3] Little additional change in the length of the fibers occurs.

Measurements of the dichroism in the spectrum of oriented DNA samples may be interpreted in terms of structural changes occurring upon dehydration and rehydration. The dichroic ratio (R) may be calculated for a band using the definition $R = A_{\parallel}/A_{\perp}$ where A is the absorbance of the band with the electric vector of the radiation polarized parallel ($\parallel$) and perpendicular ($\perp$) to the direction of molecular orientation of the DNA film. Values of R greater or smaller than unity are indicative of the systematic orientation of molecular subgroups and changes in R may arise from either disordering of the molecular groups or a systematic change in the orientation of the groups.

The 1660 cm.$^{-1}$ band in the infrared spectrum and the 2600 Å. band in the ultraviolet spectrum of NaDNA are useful in this respect. The transition moments connected with these absorptions lie in the plane of the purine–pyrimidine bases[16,17] for reasons of symmetry. Thus a very low value of R would be expected for these bands if measurements were made on a well oriented film at 92% r.h. As r.h. is lowered, either a systematic change in the tilting of base-pairs or a random change in the orientation of bases might occur in DNA. If the tilting is systematic, *i.e.*, if the angle θ changes equally for each base-pair in the helix, equations may be derived relating the measured dichroic ratio to the angle θ.

The following model has been used for this purpose. It was assumed that the DNA molecules were long and rod-like and that the film was a planar sample containing a fraction f of perfectly oriented molecules and a fraction $(1 - f)$ of molecules randomly oriented in the plane of the sample. It also was assumed that the absorption band arises from a single transition moment which lies in the plane of the base pair and makes an angle γ with the helix axis. Since the absorbance is proportional to $(\mathbf{E}\cdot\mathbf{M})^2$ where $\mathbf{E}$ is the electric vector of the polarized radiation field and $\mathbf{M}$ is the transition moment, equation 1 is obtained[18]

$$\tan^2\gamma = 2\frac{(1+f) - R(1-f)}{R(1+f) - (1-f)} \qquad (1)$$

This may be rewritten as

$$= \frac{(R-1)\cos^2\gamma + {}^1/_2(R-1)\sin^2\gamma}{(R+1)\cos^2\gamma - {}^1/_2(R+1)\sin^2\gamma} \qquad (2)$$

These equations may be used as follows. A film is equilibrated at 92% r.h. and is assumed to be in the B form ($\theta = \gamma = 90°$). The measured R then is used in equation 2 to calculate f. R values measured at lower r.h. are then used with f and equation 1 to determine other values of γ. The relation between γ and θ is dependent upon the angle between $\mathbf{M}$ and the line about which the base-pair rotates as it tilts (line of tilting). If $\mathbf{M}$ is perpendicular to the line of tilting, then $\gamma = \theta$. If $\mathbf{M}$ is parallel to the line of tilting, then $\gamma = 90°$ and R remains constant no matter what value θ assumes. Thus the relation $\theta \leqslant \gamma$ always holds and the value of γ will be equal to the maximum possible θ consistent with an observed R. If an absorption band arises from transitions connected with several different non-parallel transition moments lying in the plane of the base-pair, the relation between θ and γ becomes $\theta < \gamma$ and other considerations are unchanged. It should be recalled that these relations are valid only if a *systematic tilting* of the base planes occurs.

X-Ray diffraction results for fibers of NaDNA indicate that between 85 and 80% r.h. θ changes from 90° in the B configuration of DNA to 70° in the A configuration. The curves of R *vs.* r.h. for the 1660 cm.$^{-1}$ and the 2600 Å. bands show a slight increase between 92 and 75% r.h. It is very unlikely that both transition moments lie parallel to the line of tilting of the base-pair since these are independent phenomena with no reasons of symmetry to make them coincide. Thus the near constancy of R values from both bands indicates that the bulk of the sample remains in the B configuration at 75% r.h.[19] although some small fraction of the helices may have changed to the A configuration or may have become disordered. The small increase in R observed upon going from 92 to 75% r.h. is also consistent with a small systematic tilting in all helices. It is likely that the rigid substrate to which the DNA films adhere places a constraint on the contraction accompanying the transition from the B to the A form, thus preventing or largely preventing the transition from occurring.

The infrared evidence for the transition from B to A configuration previously presented[11] rests on the presence of two plateaus above 70% r.h. in the curves of the dichroic ratios *vs.* r.h. for the bands at 1660 and 1053 cm.$^{-1}$ and frequency *vs.* r.h. for the band at 1220 cm.$^{-1}$. Our data show no evidence of such plateaus.

The sharp increase of the dichroic ratios as r.h. is lowered from 75 to 55% indicates that a considerable change in the structure of DNA occurs in this range. The observed values of R close to unity (0.75 to 1.0) indicate that either a large systematic tilting of the base-pairs occurs or that individual bases tilt through large angles but in a non-systematic way. Systematic tilting is excluded since the above R values lead to θ equal to or smaller than 60 to 54°. Values of θ as small as this are unlikely on steric grounds and are incompatible with the value of 70° obtained for the A configuration by X-ray crystallographic methods. Thus below 55% r.h. the bases have been displaced from their former positions in the B configuration and lie in a disordered array.

Ultraviolet Hypochromism.—It is well known[20-22] that the absorbance of the band at 2600 Å. in the ultraviolet spectrum of DNA in aqueous solution is about 30% lower than that of a similar band in a solution containing an equivalent concentration of nucleotides. Such an increase in the absorbance occurs when DNA in solution is denatured by thermal or chemical means.[21] This denaturation involves a change in state from two-strand helices to single-strand random coils, in which both the stacking and the pairing of the bases as in the Crick and Watson scheme[23] are largely lost.[24] Thus high values of absorbance at 2600 Å. may be

(16) M. Kasha, "Light and Life," McElroy and Glass, Ed., Johns Hopkins Press, Baltimore, Md., 1961, p. 31.

(17) G. R. Wilkinson, W. C. Price and E. M. Bradbury, *Spectrochim. Acta*, **14**, 284 (1959).

(18) Expressions similar to equation 1 but for a somewhat different model have been reported by R. D. B. Fraser, *J. Chem. Phys.*, **21**, 1511 (1953). An equation which is a rearrangement of the above also was derived by Fraser and quoted by E. M. Bradbury, Ph.D. Thesis, University of London, 1959.

(19) A fiber of the NaDNA used in this work was kindly X-rayed for us by Professor Wilkins of King's College, London, and was found to be in the A configuration at 75% r.h.

(20) R. Thomas, *Biochem. Biophys. Acta*, **14**, 231 (1954).

(21) D. O. Jordan, "Chemistry of Nucleic Acids," Butterworth, London, 1960.

(22) W. Rhodes, *J. Am. Chem. Soc.*, **83**, 3609 (1961).

(23) F. H. C. Crick and J. D. Watson, *Nature*, **171**, 737 (1953).

(24) P. Doty, H. Boedtker, J. R. Fresco, B. D. Hall and R. Haselkorn, *Ann. N. Y. Acad. Sci.*, **81**, 693 (1959).

used to infer that either the stacking or the pairing of the bases, or both, have been lost.[24,25]

The form of the curves of the absorbance *versus* r.h. for solid DNA (Fig. 3) is similar to the curves of absorbance *versus* temperature or concentration of the denaturing agent, derived from denaturation studies in solution.[21] A 1.3 to 1.5-fold increase in absorbance occurs over relatively narrow limits of r.h., temperature, or concentration. We are thus led to conclude that the structural changes occurring in solid DNA between 75 and 55% r.h. involve a complete or nearly complete loss of systematic base-stacking and probably also of base-pairing and are in this respect analogous to the denaturation of DNA in solution, except that in solution the sugar–phosphate chains uncoil whereas this process is unlikely to occur in the solid.

A structure in which base stacking order is lost and sugar–phosphate chains are distorted from their former positions is in agreement with both infrared and X-ray results. The broadening of certain infrared bands at low r.h.[12] suggests that chemically equivalent groups in the DNA polymer differ in their surroundings or conformation. The X-ray studies of Franklin and Gosling[3] on DNA fibers indicate a highly disordered structure below 55%. The present conclusions about the structure of films of DNA are consistent with their findings and we believe that the configuration of DNA at low r.h. is the same in both films and fibers.

That the sugar phosphate chains do not uncoil is in agreement with the observed reversibility of the transition since in this way the dissociated pairs are kept in register and may rapidly re-match upon rehydration.

Stability of the Helical Configuration of DNA.—It has been pointed out recently that hydrogen bonding between the base-pairs may not provide the stability of the helical structure of DNA in aqueous solution.[26-28] The results of the denaturation studies employing titrations to acid pH provide evidence that the DNA configuration remains intact while a large fraction of basic sites in the purine–pyrimidine pairs are protonated.[27] This shows that the helical structure of DNA persists even with many of the inter-base hydrogen bonds destroyed. This is not surprising if note is taken of the fact that the long hydrogen bonds postulated for the base-pairing scheme[29] would be expected to be quite weak. If these N–H.....N and N–H.....O bonds indeed have lengths of nearly 3.0 Å. assigned to them,[29] they would be expected to be weaker than the O–H....N, O–H....O and N–H.....O hydrogen bonds formed between the bases and water in the random coil configuration, and would be much weaker than the strong water–water hydrogen bonds in liquid water. Thus something else must account for the stability of the double helix in aqueous solution.

Herskovits, Singer and Geiduschek[28] have pointed out that the helical structure of DNA is stable in solution since this structure allows the maximum number of relatively strong water–water interactions to occur. The stacking of the aromatic bases gives rise to a configuration in which the relatively non-polar parts of each macromolecule are clustered together while the ionic groups remain in contact with water in analogy to a detergent micelle. The disordering of DNA upon drying provides evidence that these interactions do stabilize the ordered B configuration in solution and in the highly hydrated solid state.

The helices in solid DNA are hydrated with about 20 water molecules per nucleotide at 92% r.h.[14] As the r.h. is lowered, water molecules are removed from between the helices. At 80% r.h. all the hydration sites of the DNA molecule are still filled,[12] and the conditions for the stability of an ordered helix are still satisfied. Upon further drying, 4 to 5 water molecules per nucleotide are desorbed between 75 and 55% r.h.,[14] most of which formerly filled the grooves of the helices, while those attached to the phosphate groups remain.[12] The fact that the removal of relatively few water molecules is accompanied by the disordering of the base-pairs supports the hypothesis that the B configuration is stabilized by interaction between water molecules. The process is reversed easily by increasing r.h., as would be expected, since bases are kept in register in the disordered form. Since the phosphate groups remain largely hydrated between 75 and 55% r.h., the energy contribution from the initial hydration of the phosphate groups cannot be a factor in determining the stability of the helix.

The removal of the water molecules from the grooves of the helix would not be expected *per se* to weaken the inter-base hydrogen bonds. Thus, if the inter-base bonds remain intact below 55% r.h., they would not contribute to the stability of the helix. Even if these inter-base hydrogen bonds are broken below 55% r.h. (as seems likely), they surely would be replaced by new bonds between base-sites and the remaining water molecules or other parts of DNA. These bonds would be of about the same strength as the original inter-base bonds they replace.[30] It therefore seems unlikely that inter-base hydrogen bonds play an important part in stabilizing the DNA helix either in solution or in solid DNA at high r.h.

Acknowledgment.—We thank Dr. G. R. Bird for lending us the wire-grid polarizer, Mr. A. Makas for the ultraviolet polarizer, Dr. E. M. Bradbury for providing us with a copy of his thesis, Dr. E. R. Blout and Dr. R. Langridge for interesting discussions, Dr. P. F. Davison for helpful advice and Dr. M. H. F. Wilkins for recording the X-ray diffraction of our DNA and for interest in this work.

(25) At the present time the question of the cause of the hypochromism is unresolved. The effect has been thought to arise from *base-pairing*[24] (through the distortion of the electron distribution caused by hydrogen bonding), or from *base-stacking* (through the dispersion interactions of the π-electrons of adjacent bases).[22] The present discussion is not sensitive to the above alternatives since in the solid structure a loss of pairing is likely to be accompanied by a loss of stacking and *vice versa*.

(26) J. M. Sturtevant, S. A. Rice and E. P. Geiduschek, *Discussions Faraday Soc.*, **25**, 138 (1958).

(27) E. P. Geiduschek, *J. Polymer Sci.*, **31**, 67 (1958).

(28) T. T. Herskovits, S. T. Singer and E. P. Geiduschek, *Arch. Biochem. Biophys.*, **94**, 99 (1961).

(29) L. Pauling and R. B. Corey, *ibid.*, **65**, 164 (1956).

(30) We interpret the bands at 3206 and 3353 cm.$^{-1}$ in the spectrum of dry NaDNA (ref. 12) as arising from N–H....O and N–H....N groups having N....O and N....N distances in the range of 2.92 to 3.02 Å. as obtained from the plot of K. Nakamoto, M. Margoshes and R. E. Rundle, *J. Am. Chem. Soc.*, **77**, 6480 (1955). The only evidence of stronger hydrogen bonds in dry DNA is the very weak band at 2800 Å., which corresponds to a negligible fraction of the total number of hydrogen bonds existing in the disordered state. Thus we conclude that the bulk of the hydrogen bonds which exist in the solid denatured state are of equal length, and therefore of nearly equal strength, to those formerly existing in the B configuration.