SPECTROPHOTOMETRIC STUDY OF THE PROTONATION OF UNDENATURED DNA

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The denaturation of DNA by controlled treatment with mild acid generally resembles the heat denaturation and results in the destruction of the characteristic two-stranded helical structure. This is shown by the marked decrease in viscosity, by the hyperchromic effect, and by the increased availability of the bases for acidic and basic titration (for reviews see Peacocke, 1957; Sturtevant, et al., 1958). Light scattering indicates no significant decrease in molecular weight for thymus DNA (see, however, Cavalieri, et al., 1959). However, at 0° C, calf thymus and herring sperm DNA can be brought to a pH of 2.6 with the extent of protonation being ca. two protons per four P, without denaturation, as evidenced by the reversibility of the titration curves and the unchanged and high intrinsic viscosity after reneutralization (Peacocke and Preston, 1958; Geiduschek, 1958; Cavalieri and Rosenberg, 1957).

We have made a spectrophotometric study of the acidification of calf thymus DNA over the range of temperatures, 0°-30°, and ionic strengths, 0.1-0.5 M (NaCl), in order to obtain further information about the sites of protonation and the effects of protonation on the ordered structure of native DNA.

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Fig. I displays the essential results. As the pH is lowered, resulting as we shall see in the protonation of DNA, the absorbance at 257 mμ falls whereas that at 280 mμ increases. As more acid is added, a point is reached where the light absorption at all wave lengths rises rapidly with decreasing pH and the ratio $\epsilon_{280}/\epsilon_{265}$ continues to increase.

It should be recalled that of the three basic nucleosides, cytidine (pK_a=4.2) shows a decrease in ϵ_{257} and an increase in ϵ_{280} with protonation, with an isosbestic point at 265 mμ. The spectrum of adenosine (pK_a=3.5) is little affected by protonation, whereas the weakest base, guanosine (pK_a=1.6), shows significant changes only below 260 mμ and above 280 mμ.

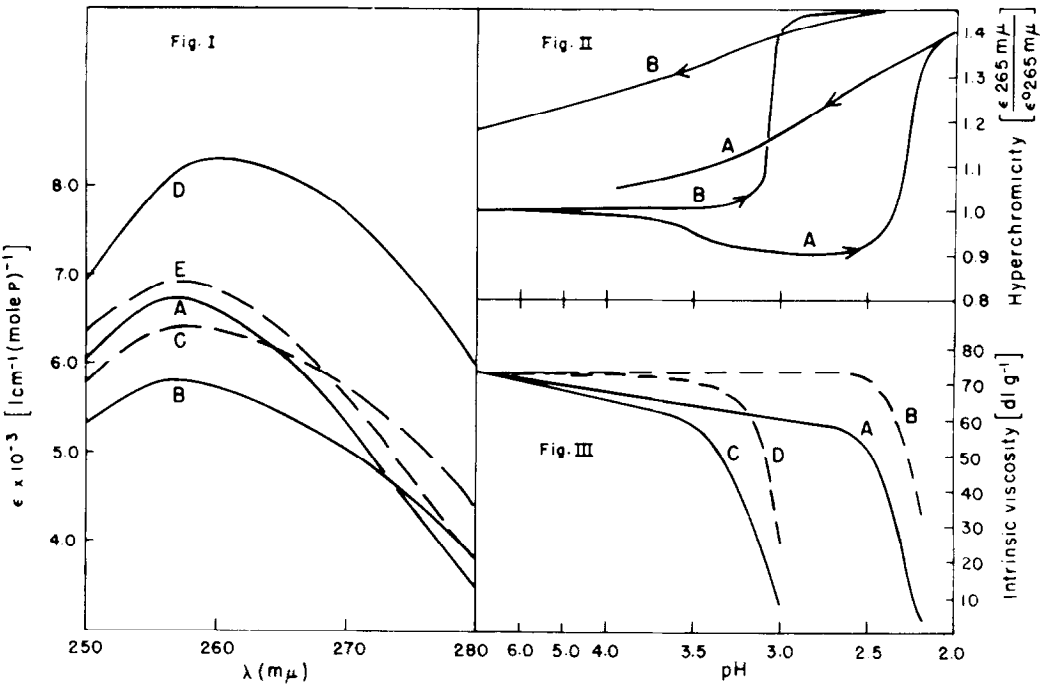

Fig. I. Absorption spectra of DNA at 0.0(± 0.2)°C in 0.1 M NaCl. A, original DNA, pH 6; B, pH 2.80; C, pH 2.42; D, pH 2.25; E, reneutralized, pH 8.

Fig. II. Hyperchromicity vs. pH on forward and back titrations at 0° (A) and 30° (B).

Fig. III. Intrinsic viscosity in acidic and reneutralized form vs. pH. A, 0° at pH indicated; B, 0°, reneutralized from indicated pH; C, 30°, at pH indicated; D, 30°, reneutralized from indicated pH.

The general increase in absorption in the more acid solutions is a hyperchromic effect, indicating denaturation, and is well known from previous studies of the acid and heat denaturation. The changes in spectra before denaturation, i.e., below pH 2.59 at 0° and pH 3.32 at 30°, are like the spectral changes on protonation of cytidine. Since cytidine and adenosine are stronger bases than guanosine, we attribute these changes to the protonation of cytosine in the DNA. We believe that the site of this protonation is the carbonyl oxygen of cytosine and that protonation here need not disrupt the Watson–Crick pattern of hydrogen bonds. Briefly, the principal evidence for this hypothesis is the shift in spectrum of cytidine towards the red on protonation. Protonation of a sigma electron pair on the N-1 ring nitrogen would have relatively little effect on the spectrum; protonation of the amino group would suppress the resonance of this group with the ring and shift the spectrum toward shorter wave lengths; whereas protonation of the carbonyl oxygen would increase the amount of resonance in the ring and cause the observed spectral effects. The spectra of most model compounds confirm the above statements. There are however some anomalies and exceptions, and we regard our hypothesis as being probable, but not certain.

By assuming that the shapes of the absorption curves of the bases are the same in native DNA as for the free bases, we calculate from the $\epsilon_{280}/\epsilon_{265}$ ratios, plus the fact that thymus DNA contains 25 mol % cytosine, that at pH 2.7 at 0° C (μ=0.1 M) about 90% of the cytosine is protonated. At this point, according to pH–titrations, there are about 1.7 H^+ per 4P atoms (in general agreement with the Peacocke and Preston titrations). The intrinsic viscosity of the reneutralized material is the same as that of the starting material in agreement with Geiduschek, and Cavalieri and Rosenberg. It is noteworthy that the isosbestic point for protonation of the undenatured material shifts from 265 mμ at 30° to 274 mμ at 0°.

The general hypochromic effect in native DNA is not understood, but it appears to be due to the compact packing of the bases. The spectra then indicate that this compact packing is not destroyed by protonation up to the denaturation point.

We are tempted to conclude that at 0° the molecule is stable with one proton on each adenine and one on each cytosine, but that denaturation occurs when protons are added to the guanine partners of some of the cytosines.

The pH of denaturation behaves in the expected way as a function of temperature and ionic strength (Table I). The optical changes on denaturation (Fig. II) agree with those reported previously (Lawley, 1956) and indicate a partial recovery of order on reneutralizing the denatured material.

Table I

Denaturation pH

T μ	0° C 0.1	0° C 0.5	10° 0.1	20° 0.1	30° 0.1	30° 0.5
pH_{id}	2.59	2.44	2.73	3.13	3.32	2.92
% CyH^+	90	83	90	80	75	-
$H^+/4P$	-	-	1.6	1.5	1.3	-
pH_{50}	2.25	2.11	2.57	2.79	3.07	2.69
% CyH^+	95	91	89	87	87	-
$H^+/4P$	-	-	2.0	2.0	1.9	-

pH_{id} is pH of incipient denaturation; pH_{50} is pH for 50% denaturation; % CyH^+ is % cytosine protonated at these points.

By controlling the changes in either pH or temperature, it is possible to produce partial denaturation. This is clear evidence for heterogeneity of calf thymus DNA in agreement with the observations on

its heat denaturation and density-gradient centrifugation (Doty, et al., 1959).

The table shows that the effect of ionic strength is principally an effect on the titration curves. The higher the ionic strength, the lower the proton affinity of the bases because of the neutralization of the negative charge on the peripheral phosphate groups by the ionic medium. At an equal degree of protonation the sensitivity to denaturation is about the same at high salt and low salt, indicating that the external salt does not greatly affect the repulsions between positively charged base groups.

Calf thymus DNA, prepared by a modified Mirsky-Pollister procedure, was supplied by the Nutritional Biochemicals Corporation: phosphorus 7.3% of bottle weight, protein, 1-3%; $\epsilon_{260}=6.58 \times 10^3 \ell\ cm^{-1}$ (mole P)$^{-1}$; $[\eta] = 73 d\ell g^{-1}$. Sedimentation patterns of the acid-denatured, reneutralized material indicated a small amount of hydrolysis to fragments of lower molecular weight, but no significant formation of nucleotides.

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