pH TITRATION STUDIES OF THE ACID DENATURATION OF CALF THYMUS DEOXYRIBONUCLEIC ACID

Thesis by

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ABSTRACT

The acid denaturation of calf thymus deoxyribose-nucleic acid (DNA) has been studied both by pH and spectrophotometric titration in the temperature range 10 - 30°C and at ionic strengths 0.1 F and 0.5 F. The number of hydrogen ions bound by the DNA at any given pH increased with increasing temperature and decreased with increasing ionic strength. The number bound at any given pH was greater after denaturation, A model for acid denaturation is proposed. The site of proto-nation of cytosine is discussed.

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INTRODUCTION

Since the genetic continuity of species has been recognized, the method by which living organisms transmit specific characteristics to their progeny has been the object of much scientific investigation. Although at present our knowledge of this subject is far from complete, much is now known about the mechanism of heredity. F. Griffith (1) in 1928 carried out experiments in which a pure strain of Pneumococcus of one type was caused to undergo a relatively large percentage of transformations into a Pneumococcus of a second type by being allowed to grow in the presence of heat killed cells of that second type; these transformed organisms retained and transmitted their newly acquired characteristics. This gave rise to a new field of microbiology, transformation studies, and firmly established the fact that there exists in living cells some active agent, which under the proper conditions can induce specific inheritable changes. Another stride toward understanding the mechanism of heredity was made when, in 1944, Avery, Mac Leod, and McCarty (2) published the results of a careful investigation, establishing unequivocally that the active agent in transformation is deoxyribosenucleic acid, or DNA. They also reported that the transforming agent could be inactivated by heat, acid, or the action

of certain enzymes. All such inactivations were termed "denaturation." Since then, the physicochemical, as well as biological, properties of DNA have been studied intensively. The major contribution to our understanding of DNA since 1944 was the determination of its structure by Watson and Crick (3) in 1953, as two polynucleotide chains, held together by hydrogen bonds between the purine and pyrimidine rings, in the form of a double helix about a common axis. Numerous workers (4,5,6,7,8) have now proposed that those physicochemical treatments which cause "denaturation" of DNA irreversibly change its structure and/or chemical composition. Thus it has been shown that acidification below a critical pH, which depends on the experimental conditions, results in a dramatic decrease in viscosity and radius of gyration, and a uniform increase in absorption of ultraviolet light in the region 2200 Å - 3100 Å. Upon back titration a hysteresis is observed. On the basis of the Watson - Crick structure these changes are interpreted as indicating that as the pH is lowered, the DNA molecules become ionized, and when a critical degree of ionization is exceeded, the structure changes from its natural, active, helical form to an inactive randomly coiled form. There has been some disagreement, however, about the reversibility of acid denaturation (9,10, 11,). It has been the object, therefore, of this inves-

tigation to carry out pH titrations of DNA and to determine the dependence of the pH of denaturation, and the pK_a's of the titrable groups upon temperature and ionic strength. The results of the titrations will be correlated with spectrophotometric titrations carried out by a co-worker. In addition, the site of binding of hydrogen ions by cytosine will be discussed in relation to the spectral changes it undergoes as a function of pH.

EXPERIMENTAL

The sodium salt of calf thymus DNA, which had been prepared by a modified Mirsky - Pollister procedure, was obtained from Nutritional Biochemicals Corporation. The DNA was weighed exactly at room temperature and ambient humidity, and allowed to swell in a few ml. of standard sodium chloride solution in a zero degree cold room with occasional gentle stirring to disperse the lump of polymer. Care was taken to avoid high shear gradients in this step. When this concentrate became homogeneous it was transferred to a volumetric flask and diluted. After stirring overnight in the cold, the solution was diluted to the mark and stored in the cold room.

The concentration of DNA solutions was determined by phosphorus analysis and spectrophotometry. The amount of contaminant protein was determined by the

method of Lowry, et al (12), using bovine serum albumin as a standard. The procedure follows:

Reagents

Reagent A: 0.10 V F sodium hydroxide containing 2 weight percent sodium carbonate.

Reagent B: A 0.5 weight percent solution of copper sulfate pentahydrate containing one weight percent sodium or potassium tartrate.

Reagent C: Folin Reagent, diluted with water to 1.0 V F in hydrogen ion.

Procedure

To a sample containing 5 - 100 micrograms of protein in 0.2 ml. or less, add one ml. of a freshly made solution containing 50 ml. of reagent A and one ml. of reagent B. Mix the solution well and let it stand for ten minutes at room temperature. Then add 0.10 ml. of reagent C rapidly while stirring. After thirty minutes the absorbtivity is determined in a spectrophotometer. For concentrations of 5 - 25 micrograms per ml., readings should be taken at 7500 Å. For more concentrated solutions, 5000 Å is more suitable.

Reagents

Reagent grade chemicals were used in all preparations. All volumetric apparatus was calibrated prior to use.

Approximately 0.1 VF (volume formal) carbonate free sodium hydroxide stock solutions were prepared and standardized against potassium hydrogen phthalate, with phenolphthalein as an indicator. Approximately 0.1 VF hydrochloric acid stock solutions were prepared and standardized against the sodium hydroxide solution.

All aqueous solutions were prepared with equilibrium water, containing sodium chloride to maintain a

desired ionic strength.

Apparatus

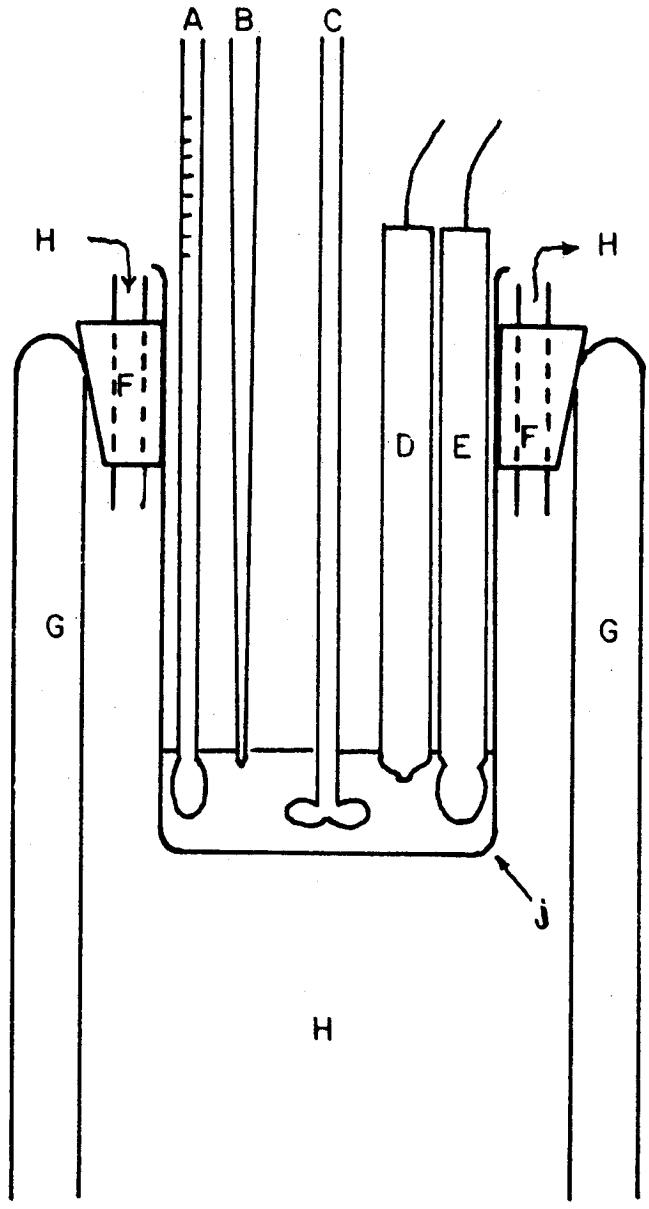
The titration cell assembly, shown in Fig. 0, consisted of a 50 ml. beaker supported in a dewar flask by a large rubber ring, through which glass tubes passed to allow flow of thermostat fluid. Temperature of the solution being titrated was thus maintained within ±0.2°C of the desired value. The titrant was delivered from a 5 ml. microburette by means of a special capillary tip.

pH measurements were made with a Beckman Model G pH meter, equipped with large external electrodes, and were reproducible to within ±0.01 units except near neutrality where the slopes of titration curves were very large.

Viscosity measurements were made with an Ubbelohde type capillary viscometer modified according to Schneider (13). The instrument was constructed with three bulbs, and a 150 cm. long capillary of radius 0.50 mm. wound in a helix. The maximum shear gradients for the three bulbs were approximately 220, 150, and 90 $sec.^{-1}$.

All spectrophotometric measurements were made with a Beckman Model DU spectrophotometer. For measurements in the ultraviolet region, a hydrogen lamp and quartz cells were employed. Corex cells and a tungsten lamp were used for measurements in the visible region.

Figure O



A. Thermometer

B. Burette tip

C. Stirrer

D. Beckman calomel electrode

E. Beckman glass electrode

F. Rubber support

G. Dewar flask

H. Thermostat fluid

j. 50 ml. beaker

Procedure

Standard solutions of hydrochloric acid and sodium hydroxide were prepared by diluting an aliquot of the standardized stock solution in a volumetric flask. The pH meter was allowed to equilibrate for about one hour, and was then adjusted with Beckman pH7 buffer solution and pH4 buffer solution. An aliquot of equilibrium water of the desired ionic strength was pipetted into the titration cell and was titrated to approximately pH 2.5. This blank was then removed from the cell, which was cleaned and dried. An equal aliquot of DNA solution was introduced into the cell, titrated to approximately the same pH, and then back titrated to neutrality. The titrant was added slowly with stirring to avoid local high concentrations of acid or base. After each titration the pH meter was checked against the buffer solutions again. If any drift was observed it was assumed to be linear in time and appropriate corrections to observed pH values were made. The viscosity and ultraviolet spectrum of the titrated solution was then measured.

Initially, titrations of DNA solutions approximately 0.0001 M in phosphorus were attempted. However, it was found that the separation of the titration curve of the blank from that of the DNA solution was of the same order of magnitude as the uncertainty in a pH measurement, $\pm$0.01 units. Furthermore, solutions 0.01 M or

greater in phosphorus were found to be too viscous to permit rapid mixing of solution and titrant. Solutions about 0.001 M in phosphorus allowed rapid mixing, and gave a separation of forward and back titration curves sufficient to determine the change in the number of hydrogen ions bound to within $\pm$ 10%. All experiments were done on solutions of this concentration.

RESULTS AND DISCUSSION

Results

Titrations were carried out at 10, 20, and 30°C, and at ionic strengths of 0.1 and 0.5. Attempts to titrate at 0°C were thwarted, since it was impossible to equilibrate the pH meter. This was probably due to very high impedance of the glass electrode at this temperature.

The total number of hydrogen ions bound by the DNA at a given pH was determined graphically from the titration curves by subtracting the number of moles of hydrogen ion added per ml. (total volume) to the blank from the number of moles added to the DNA solution. This method gave accurate results except at the lowest pH values where slopes of the titration curves were nearly zero, making graphic measurements very difficult.

In addition to the author's pH titrations, another graduate student, Mr. W.F. Dove, carried out

spectrophotometric titrations of solutions at the same temperatures and ionic strengths. The four nucleosides of DNA, which are present in calf thymus in approximately equal amounts, are cytidine, adenosine, guanosine, and thymidine, and have pK_a's of 4.22, 3.45, 1.6, and < 0 respectively, in the free forms. In the range of pH's of these titrations only the cytosine and adenine residues of the DNA are expected to bind hydrogen ion, and indeed it was observed that the solutions bound two hydrogen ions per four nucleosides at low pH. The ultraviolet absorption spectra of the nucleosides as a function of pH are well known (14). The spectrum of adenosine is essentially unchanged upon binding hydrogen ion, whereas that of cytidine shows a decrease in absorption at 2500 Å and an increase at 2800 Å with an isosbestic point at 2650 Å. It is assumed that the spectra of the nucleosides in DNA are the same as in the free forms. (See Appendix Fig. I) Therefore spectrophotometric titrations determined the amount of hydrogen ion bound by the cytosine residues, while the pH titrations determined the total amount bound by the DNA molecules.

Table I

The physicochemical properties of the solutions investigated are summarized. Molar absorptivities and intrinsic viscosities are given for the solutions before and after titration, where available.

Figs. 1 - 3

Plots of the moles of hydrogen ion bound per four gram atomic weights of phosphorus in the DNA solution vs. pH. under various conditions:

Figure	Temperature of Experiment (^{o}C)	Ionic Strength of Experiment
1	10	0.1F (NaCl)
2	20	0.1F (NaCl)
3	22.5	0.5F (NaCl)

Fig. 4

Plot of the moles of hydrogen ion bound per four gram atomic weights of phosphorus in the DNA solution, titrated twice. Conditions: 20^{o}C, μ = 0.1F (NaCl).

Fig. 5

Plot of moles of hydrogen ion bound per four gram atomic weights of phosphorus in the DNA solution vs. temperature. Only the forward titrations were plotted.

Fig. 6

Plot of moles of hydrogen ion bound per four gram atomic weights of phosphorus in the DNA solution vs. ionic strength. Only the forward titrations were plotted.

TABLE I

Solution	Temperature of Exp't (°C)	Concentration of Phosphorus (moles/liter) ($\times 10^3$)	Molar Absorbtivity at 2575Å ($\times 10^{-3}$)		Intrinsic Viscosity (dl/gm.)		Ionic Strength
			Initial	Final	Initial	Final	
D-7	22.5	1.7	-	-	-	-	0.5
D-8	10.0	1.5_5	6.19	-	69.5	7.5	0.1
D-8	20.0	1.5_5	6.19	6.96	69.5	2.1	0.1
D-8	30.0	1.5_5	6.19	-	69.5	-	0.1
D-12	20.0	1.6	6.40	-	80.5	-	0.1
D-12	30.0	1.6	6.40	-	80.5	-	0.1

Figure 1

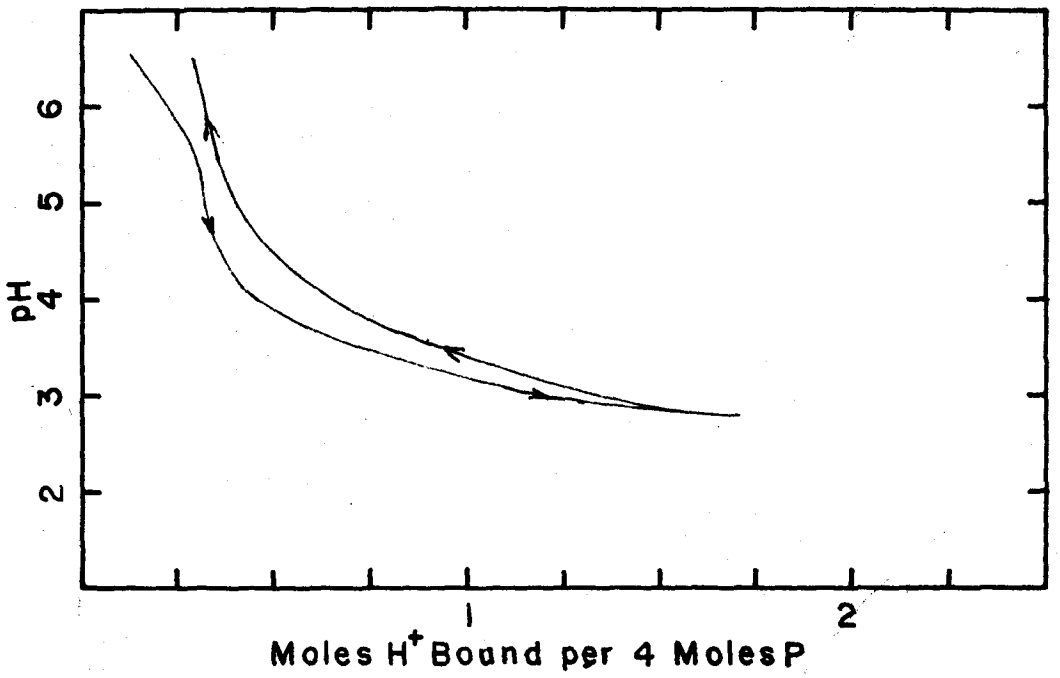
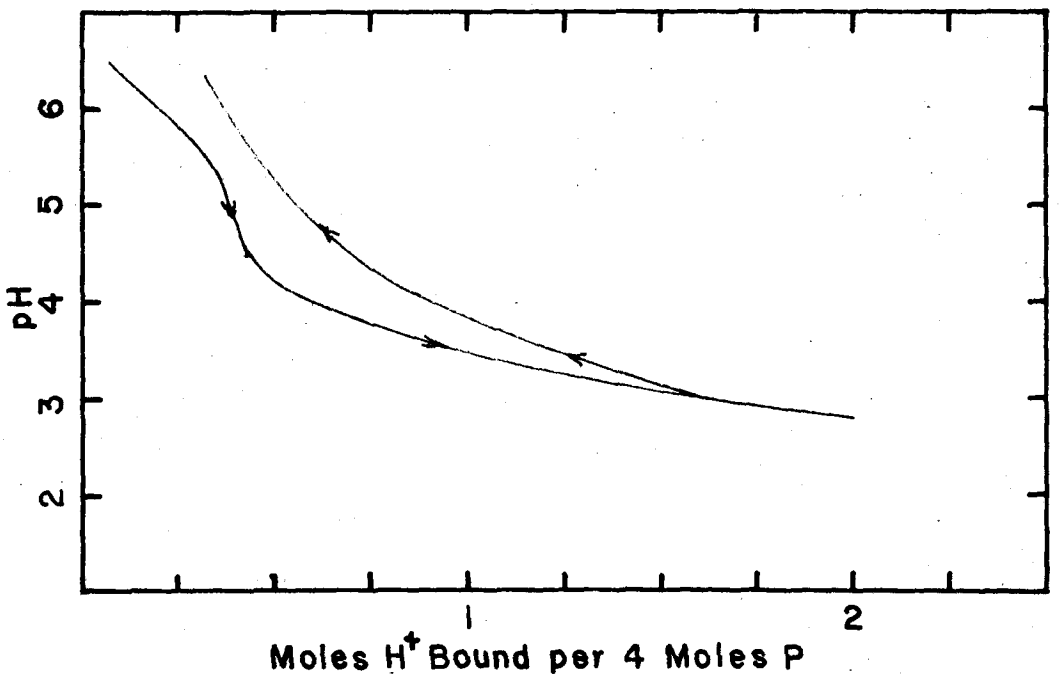


Figure 2



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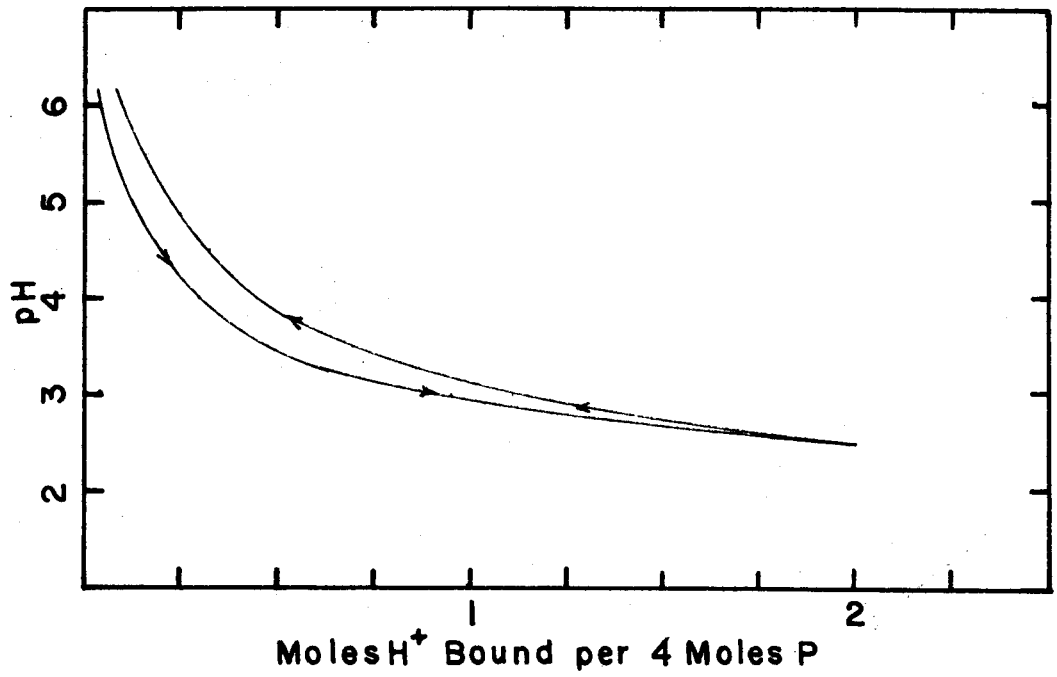


Figure 4

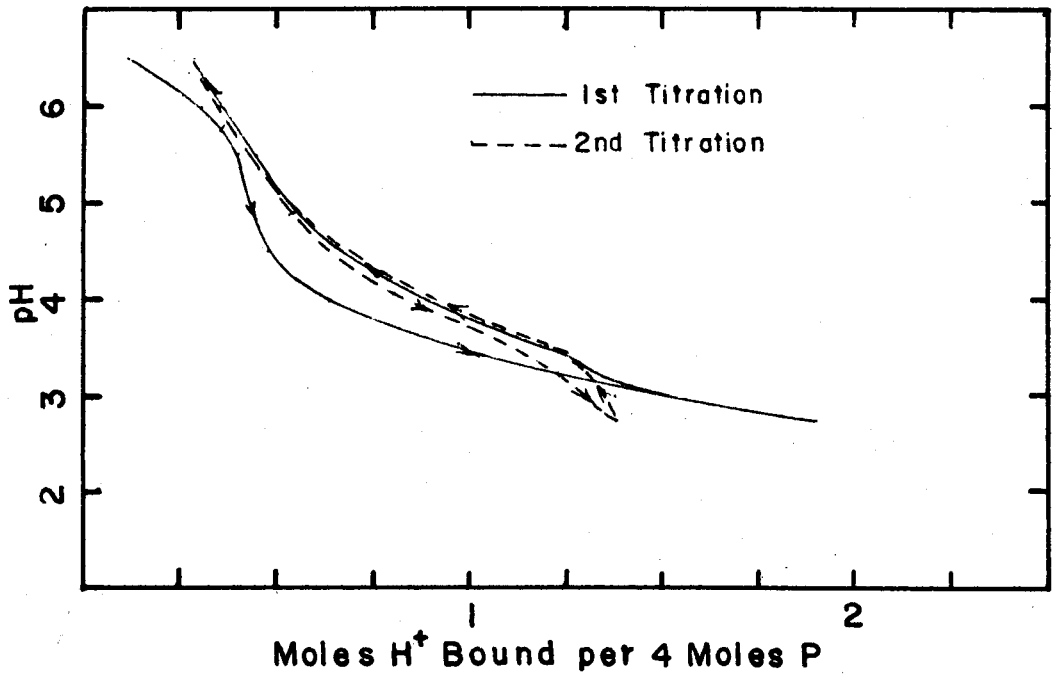


Figure 5

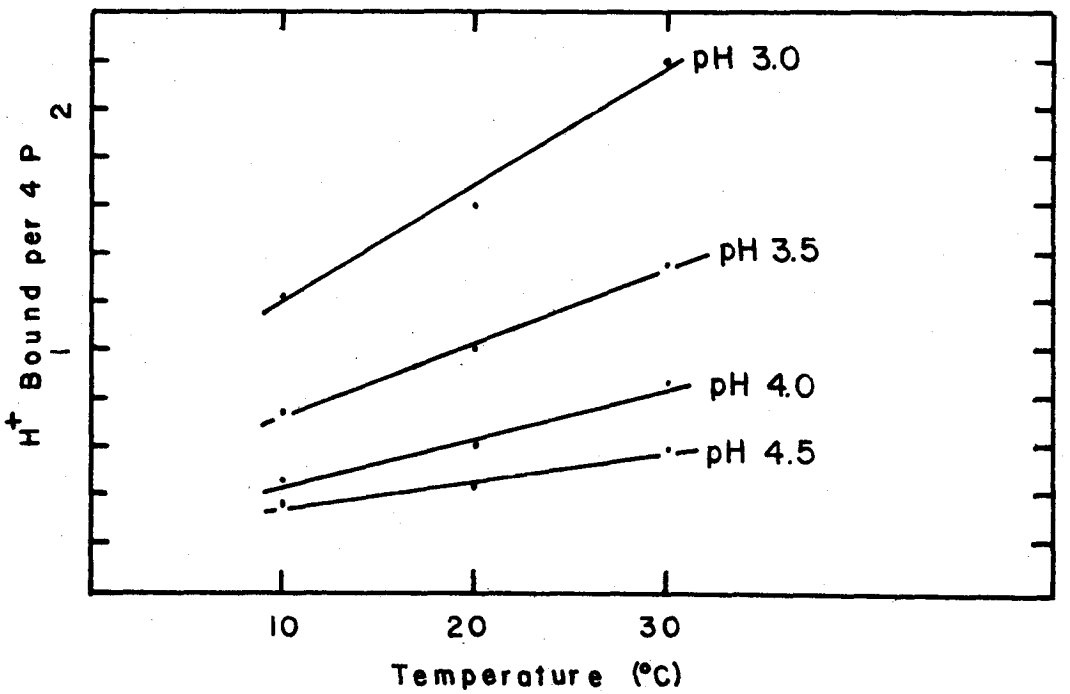
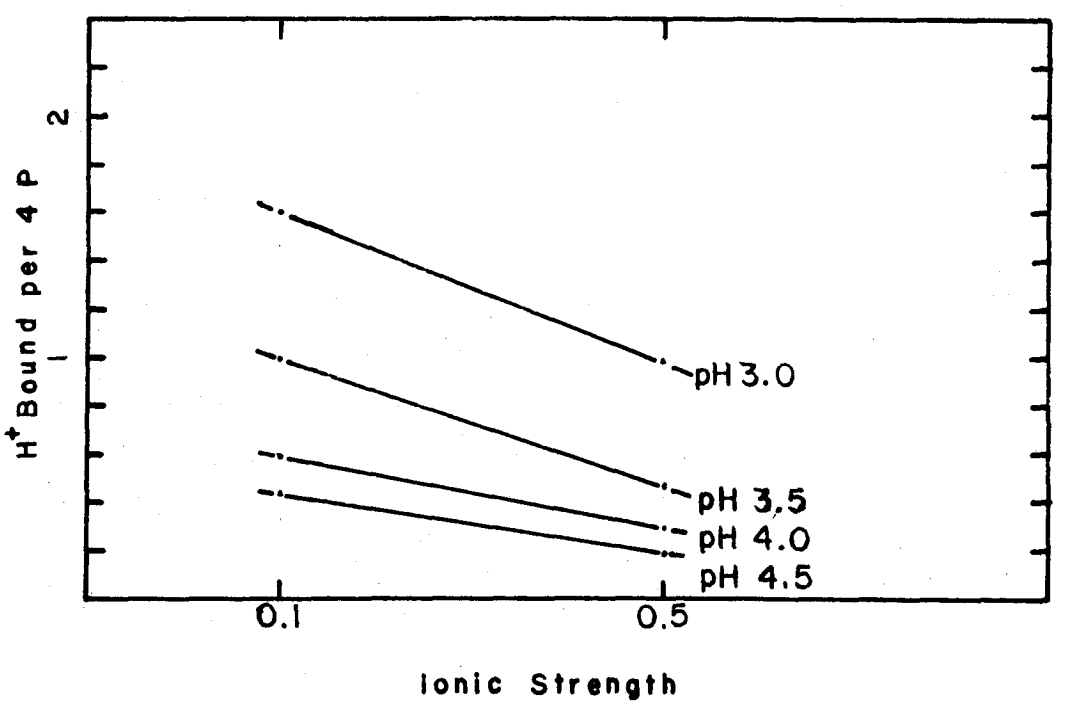


Figure 6



The effect of temperature is seen from Fig. 5. As temperature of the solution titrated increases, the number of hydrogen ions bound at a given pH and ionic strength increases.

The effect of ionic strength is seen from Fig. 6. At a given pH and temperature, the number of hydrogen ions bound increases as the ionic strength decreases.

Fig. 4 displays the results of titrating a DNA solution to beyond denaturation, back titrating to neutrality, and then repeating the procedure. It can be seen that the first titration resulted in a complete and irreversible change since subsequent titration curves were collinear with that of the back titration. The apparently anomalous behavior in the low pH range may be due to the weaknesses of the graphic method mentioned before.

Correlation of the spectrophotometric and pH titrations (see appendix, Table I) indicates that within the accuracy of the method, the pK_a's of the adenine and cytosine residues are the same in DNA, in agreement with the findings of Cox and Peacocke (15). Furthermore, it was found that the number of moles of hydrogen ion bound at the point of 50% denaturation, as determined by the hyperchromic effect (Appendix, Fig. II), is independent of temperature, being about two per four moles of phosphorus in the solution.

Discussion

On the basis of the Watson-Crick structure and the results obtained here, the following model for acid denaturation is proposed.

As the pH of a solution of DNA molecules is lowered, both adenine and cytosine begin to bind hydrogen ions. The complementary hydrogen bonding of the Watson-Crick structure requires that every link between the two chains of a molecule contain one of these two bases. From the spectrophotometric data (Appendix, Table I) the binding of hydrogen ions continues upon decreasing the pH further, to a point at which more than half (at 10°C, 80%) of the cytosine and adenine residues have hydrogen ions bound to them without any detectable change in the structure of the molecules. Since the hydrogen bonds are responsible for the stability of the double helix, it may be inferred that the binding of hydrogen ions to this extent does not alter the pattern of hydrogen bonds. Finally, at a degree of binding which is independent of temperature, the secondary structure begins to be lost, i.e. denaturation begins. However, even when the binding is complete, only 50% denaturation, as indicated by the hyperchromic effect (See Appendix), has occurred. This suggests that it may be necessary for the guanine partners of some of the cytosine residues to bind hydrogen ions in order to effect complete denaturation. At this

point the DNA molecules are collapsed randomly coiled polynucleotide chains. Whereas the native structure was an extended rodlike one, with the negatively charged phosphate groups relatively far apart, the collapsed structure is more compact, with the phosphate groups closer together. This lowers the K_a's of the cytosine and adenine residues by an electrostatic effect described in the next paragraph. On back titrating, therefore, more hydrogen ions are bound at any given pH.

The effect of ionic strength is that predicted by thermodynamics and polyelectrolyte theory. The classical thermodynamic constant for a reaction like the binding of hydrogen ions is given by

$$K = e^{-\Delta F/RT}$$

If we consider a polyion such as a DNA molecule in solution, the free energy change in such a reaction has two distinct parts. There is a free energy change associated with the formation of a chemical bond, ΔF_i, and correspondingly there is an "intrinsic" thermodynamic constant, K , associated with that. But there is also a free energy change associated with bringing a charged ion up to the polyion, ΔF_e, which is given by

$$\Delta F_e = Z e \psi$$

where Z is the number of charges on the ion, e is a unit of charge, and ψ is the electrostatic potential at the

surface of the polyion. (An increase in ψ upon denaturation accounts for the lowering of the K_a's mentioned above.) The classical expression for a polyion is therefore

$$K' = e^{-(\Delta F_i + \Delta F_e)/RT}$$

or

$$pK' = 0.4343(\Delta F_i + \Delta F_e)/RT$$

but the first term in this expression is just pK.
In solution the polyion or DNA molecule is surrounded by a cloud of counterions, which reduces the effective surface potential. As the ionic strength is increased the cloud of counterions increases, reducing the effective potential ψ, and ΔF_e. Thus increased ionic strength reduces K'.

The effect of temperature on the number of hydrogen ions bound at a given pH is related to the enthalpy change on binding, by the classical expression

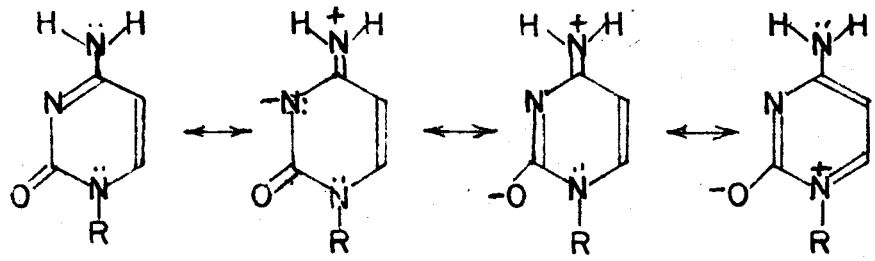
$$\left(\partial \ln K / \partial \frac{1}{T}\right) = \frac{\Delta H}{R}$$

and suggests that the ΔH of binding hydrogen ion is positive. The accuracy of the data does not justify a more quantitative conclusion.

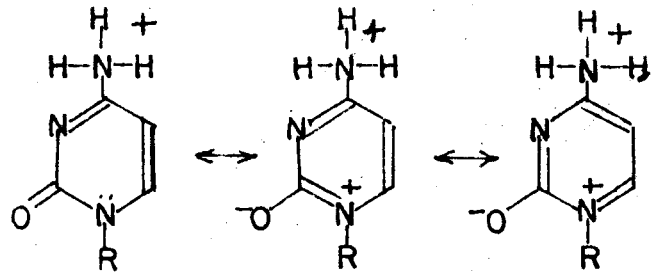
Since our results suggest that it is possible to bind hydrogen ions to DNA molecules without disrupting the hydrogen bonding, the site of binding is a critical point in the validity of the model described.

Cytidine in neutral solution exists as a resonance hybrid of structures I - IV, below. The largest contribution to the hybrid is from I, with only small contributions from II, III, and IV, due to the fact that they require a formal separation of charge in the molecule.

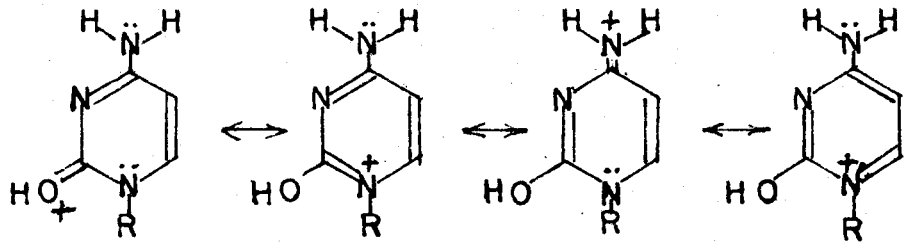
Upon binding hydrogen ion the absorption maximum increases in intensity and shifts to longer wavelengths. Such behaviour is characteristic of increased resonance in the molecule. Binding by a ring nitrogen cannot account for this change in spectrum because it would not alter resonance to such an extent. Binding by the amino group would lead to a structure having less resonance possibilities than the neutral form, and would thus be expected to shift the absorption maximum to shorter wavelengths, contrary to what is observed. These structures are V, VI, and VII below. If the hydrogen ion goes onto the oxygen, the compound would then be a resonance hybrid of structures VIII - XI below. In this case there are four equally contributing structures, which allow distribution of the positive charge over four centers, and in which there are no formal separations of charge. Only binding by the oxygen leads to structures having greater resonance than the neutral form, therefore, and we can conclude that this is what happens. Examination of the Watson-Crick hydrogen bond patterns indicates that binding at this site does not alter the hydrogen bonding.



I II III IV



V VI VII



VIII IX X XI

R = Ribose

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APPENDIX

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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*Contribution No. 2524

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.

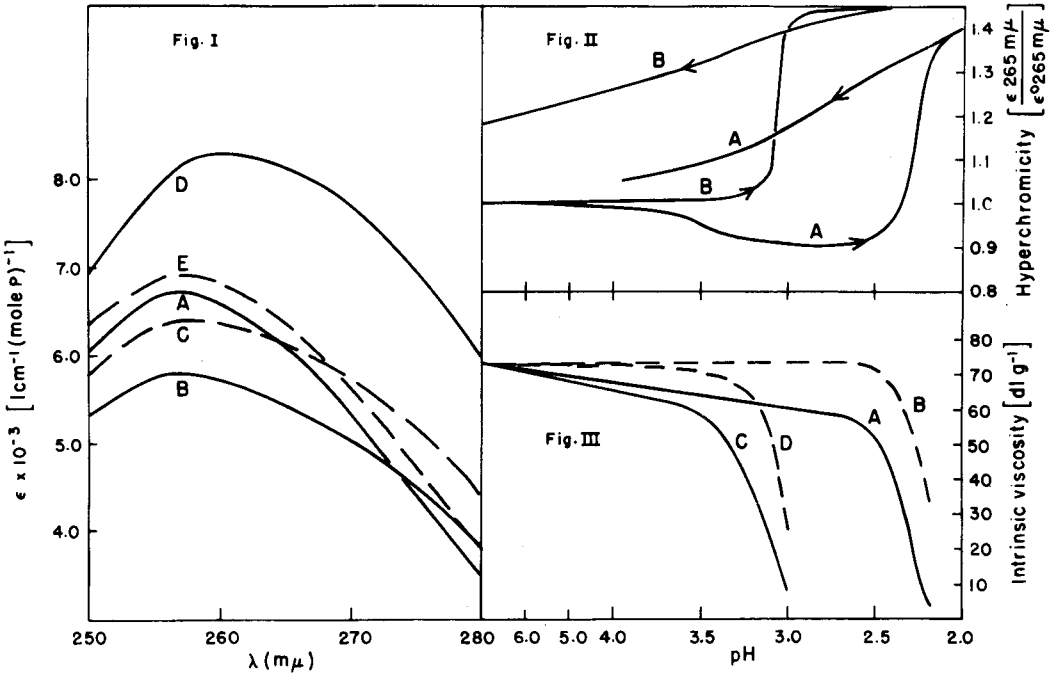

Fig. I. Absorption spectra of DNA at 0.0(± 0.2)°C in 0.1 M NaCl. A, original DNA, pH 6; B, pH 2.80; C, pH 2.42; D, pH 2.25; E, reneutralized, pH 8.

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.

We are glad to acknowledge support from the U.S. Public Health Service (Grant No. A-2145C), the technical assistance of Mr. Herbert Rice, and an NSF predoctoral fellowship for one of us (WFD). We are grateful to Professor George S. Hammond for advice and discussion about the spectra and mode of protonation of the nucleotides.

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