lated and observed $\Delta pK'$s to non-coulombic effects, such as "inductive" effects.[11]

Discussion

The Kirkwood model is a highly artificial representation of an organic ion. Any meaning attached to the parameter d must be a purely conventional one, somewhat like the "distance of closest approach" which occurs in the Debye–Hückel theory.

The variation of the *self-energy* of a point charge (within the cavity) with the distance d can be obtained from the complete equation for the work of charging.[12] The equation shows that the charge is repelled by the cavity and that its position of greatest stability is as close to the surface as possible. A similar conclusion applies to point dipoles. By analogy with the Debye–Hückel distance a, the distance d can thus be interpreted as a measure of the distance of closest possible approach of the solvent to a charged or dipolar site. This distance presumably represents in part the effective size of the atom or group of atoms on which a charge or dipole is centered. The effect of electric saturation of the solvent near a charged or dipolar site[13] may also be incorporated in the parameter d. In any event it is reasonable that, as a first approximation, the distance d should have about the same value for any charged site in a given solvent at a given temperature. It is also reasonable that a point dipole centered between carbon and halogen atoms should appear to be at a greater distance from the solvent than a discrete charge on an amino group or on a carboxyl oxygen atom.

To the extent that d is a measure of the physical distance from a charge or dipole to a water molecule it might be expected to *increase* with increasing temperature. It might also be expected to differ from one solvent to another. In this way one can account qualitatively for the failure of the Kirkwood–Westheimer theory, when used in such a way that d remains unaltered, to account for the effect of changing solvent or temperature upon acid dissociation constants.[14,15]

It was pointed out in the introduction to this paper that the principal objective of this investigation was to guide the extension of the Kirkwood model to globular proteins. The details of such an extension have been described in earlier papers.[16] The object was to compute titration curves of proteins, taking into account the interaction, as given by eq. 1, between all pairs of charges. Calculations were performed on simple models with structural features similar to those found in actual proteins. It was shown that calculated results agreeing with typical experimental observations could be obtained *only if d is placed equal to approximately 1 Å.* No reasonable assignment of distances between sites yields calculated titration curves resembling those obtained experimentally if d is assigned a value >1.5 Å. or <0.5 Å. This result is clearly in agreement with the conclusions reached in this paper concerning the location of charges in organic molecules in general.

Acknowledgment.—The author is pleased to acknowledge his indebtedness to Professors J. G. Kirkwood and F. H. Westheimer for helpful discussion of many aspects of this investigation.

(12) The quantity desired is given explicitly by the term B_{kk} of ref. 16.

(13) P. Debye, "Polar Molecules," (Chemical Catalog Co.), Reinhold Publishing Corp., New York, N. Y., 1929; F. Booth, *J. Chem. Phys.*, **19**, 391 (1951).

(14) W. F. K. Wynne-Jones and G. S. Rushbrooke, *Trans. Faraday Soc.*, **40**, 99 (1944).

(15) M. Kilpatrick and J. G. Morse, THIS JOURNAL, **75**, 1846, 1854 (1953).

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NEW HAVEN, CONN.

[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING DIVISION, CORNELL UNIVERSITY MEDICAL COLLEGE]

Studies on the Structure of Nucleic Acids. XI. The Roles of Heat and Acid in Deoxyribonucleic Acid Denaturation[1]

BY LIEBE F. CAVALIERI AND BARBARA H. ROSENBERG

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The denaturation of DNA by heat and acid has been studied by means of potentiometric titration. Titration curves have been determined at various temperatures and ionic strengths. Denaturation occurs during the steep inflection of the forward (neutrality → low pH) titration curve; the extent of denaturation depends on the proportion of this inflection which is titrated. Before denaturation begins there are some hydrogen bonds which are reversibly broken and healed. When a critical number of bonds are cleaved, denaturation sets in. This number depends on the temperature, ionic strength and the solvent system. In the presence of urea, fewer groups must be titrated potentiometrically to cause denaturation; that is, the equilibrium intact ⇄ broken bonds is shifted to the right for any given temperature and ionic strength. It is also concluded that the effects of heat and acid, when acting simultaneously, are additive.

The anomalous (irreversible) titration of deoxyribonucleic acid (DNA), found by Gulland and coworkers,[2] was fruitfully considered to be evidence for the existence of hydrogen bonds between the bases. However, it will be shown here that this evidence is indirect. That is, the increased pK'_a's which follow exposure to acid result directly from changes in the charge constellation of the molecule, which in turn depends on hydrogen bonding. Cox and Peacocke[3] recently have established that the back-titration curves, after exposure of DNA to pH 2.2 and pH 12.0, are coincident. This means that acid and alkali, although they directly break different hydrogen bonds since they ionize different

(1) This investigation was supported in part by funds from the Am. Can. Soc., National Cancer Institute, National Institutes of Health, Public Health Service (Grant #Cy3190), and from the Atomic Energy Commission (Contract #AT(30-1)-910).

(2) J. M. Gulland, D. O. Jordan and H. F. Taylor, *J. Chem. Soc.*, 1131 (1947).

(3) R. A. Cox and A. R. Peacocke, *ibid.*, 2499 (1956).

groups, produce the same pK'_a increases. Therefore the final state produced by either acid or alkali very likely contains none of the original hydrogen bonds. This final state has been called "denatured." In this condition, DNA has lost biological (transforming) activity[4] and undergone many irreversible physical property changes. Some of the latter, such as the complete loss of flow dichroism,[5] demonstrate the disappearance of the original hydrogen bonding.

The approach to the denatured state, contrary to expectation, is not gradual. We have shown here and elsewhere,[6] that irreversibility of the titration curve does not commence until a number of groups have been titrated; thereafter, irreversibility increases to a maximum over a narrow pH range. Cox and Peacocke[3] have concluded from similar experiments that hydrogen bond cleavage by acid is irreversible only when a sufficient number of adjacent bonds have been broken to permit irreversible changes in molecular configuration. Other physical measurements, such as the steep optical density rise at 260 mμ[7-9] and the sharp viscosity drop observed by Thomas and Doty,[10] have also shown sudden, discontinuous and irreversible changes with pH. It may be concluded (see, for example, ref. 5) that the denatured molecule has collapsed from its specific, helical configuration into a random one. The sudden occurrence of denaturation indicates that as long as the cumulative hydrogen bonding forces in the molecule are sufficient to overcome the forces tending to disrupt its orderly structure, the molecule remains intact; but when the former force decreases below the latter, the remaining hydrogen bonds rupture spontaneously.

Denaturation by exposure to low ionic strength[9] has been shown[5] to be exactly the same phenomenon as acid denaturation, by virtue of the dependence of the pK''_a's on ionic strength: in $10^{-4}M$ salt, approximately one-half of the basic groups are ionized at pH 7. Salt dependence was first shown potentiometrically by Lee and Peacocke[11] for both the forward and backward titration curves. The wide range ($\sim$ 4 pH units) observed for each (undenatured) base pK'_a as a function of ionic strength implies a certain charge distribution in the molecule.[8] The Watson–Crick model[12] successfully accounts for all the observations discussed above.

All the preceding work was performed at room temperatures (15° for English winters and 35° for American summers). However, heating has also been found to produce denaturation. The same precipitous changes, such as viscosity drop[13,14] and optical density increase[15] that characterize acid denaturation can be brought about merely by heating to an appropriate temperature. Furthermore, the same pK'_a shifts occur, so that after heat denaturation "forward" titration follows the back-titration curve.[16] It seems reasonable to conclude that, under conditions that do not cause degradation, heat and acid (or alkali) produce, by different mechanisms, the same, final denatured state in the DNA molecule. In fact, experiments reported below, and by Cox and Peacocke,[17] show that heat and acid can actually supplement each other to produce denaturation under conditions in which neither would do so alone. We have investigated the action of heat on DNA, through its effect on the titration curves.

Experimental

Materials.—Most of the experiments were done with two samples (D-III and D-IV)[6] of calf thymus DNA prepared, with modifications,[6] according to the procedure of Kay, Simmons and Dounce.[18] *Anal.* D-III: N, 11.00; P, 6.75; Na, 9.53; H_2O, 16.83.

The same results were obtained using another thymus sample (D-V) prepared by the same method (*Anal.* N, 12.38; P, 7.59; Na, 8.03; H_2O, 19.57) and with a thymus sample (S-II)[6] prepared by the Schwander and Signer procedure[19] (*Anal.* N, 13.42; P, 7.97; Na, 7.70; H_2O, 21.2).

DNA concentrations (on dry weight basis) of 1 mg./ml. were used for titration, 1 mg./ml. and 0.02 mg./ml. for optical density measurements, and 0.063 mg./ml. for viscosity measurements. All solutions were made by dissolving DNA in 10^{-3} M NaCl and then adjusting the salt concentration with solid salt or by dilution. The salt concentrations and ionic strengths mentioned herein include counterions from the DNA.

Continuous titrations were carried out using calomel and glass electrodes (Beckman 1190-80) which had been checked with buffers over the entire temperature range (0–75°) of interest. Diffusion from the salt bridge was checked and found insignificant under the conditions of the experiments. The Beckman Model G pH Meter and an Automatic Recording Titrator,[20] model AT-2, of similar sensitivity were both employed. Hydrochloric acid (1 and 0.1 N) or sodium hydroxide (1 and 0.1 N) were added by means of a microburet to the rapidly stirring DNA solution, whose temperature was adjusted and maintained by a surrounding bath of an appropriate liquid under reflux.

All experiments were performed after temperature equilibrium had been reached—that is, after any changes in the DNA had ceased.

Spectral measurements were made in a Beckman spectrophotometer, model DU, using quartz cells of 1 and 0.01 cm. path length.

Viscosity measurements were made in a 5-ml. Ostwald Viscometer (flow time 1.5 min. for 0.2 M NaCl at 22.3°).

Results

When DNA is titrated potentiometrically[21] at

(4) S. Zamenhof, H. E. Alexander and G. Leidy, *J. Exptl. Med.*, **98**, 373 (1953).

(5) L. F. Cavalieri, M. Rosoff and B. H. Rosenberg, THIS JOURNAL, **78**, 5239 (1956).

(6) L. F. Cavalieri and B. H. Rosenberg, *Biochim. Biophys. Acta*, **21**, 202 (1956).

(7) L. F. Cavalieri, THIS JOURNAL, **74**, 1242 (1952).

(8) L. F. Cavalieri and A. L. Stone, *ibid.*, **77**, 6499 (1955).

(9) R. Thomas, *Biochim. Biophys. Acta*, **14**, 231 (1954).

(10) C. A. Thomas, Jr., and P. Doty, THIS JOURNAL, **78**, 1854 (1956).

(11) W. A. Lee and A. R. Peacocke, *Research Suppl.*, **6**, No. 2, 15S (1953).

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

(13) S. Zamenhof, H. E. Alexander and G. Leidy, *J. Exptl. Med.*, **98**, 373 (1953).

(14) S. Zamenhof, G. Griboff and N. Marullo, *Biochim. et Biophys. Acta*, **13**, 459 (1954).

(15) J. Shack and J. M. Thompsett, *J. Biol. Chem.*, **197**, 17 (1952).

(16) R. A. Cox and A. R. Peacocke, *J. Chem. Soc.*, 2646 (1956).

(17) R. A. Cox and A. R. Peacocke, *J. Polymer Sci.*, **23**, 765 (1957).

(18) E. R. M. Kay, N. S. Simmons and A. L. Dounce, THIS JOURNAL, **74**, 1724 (1952).

(19) H. Schwander and R. Signer, *Helv. Chim. Acta*, **33**, 1521 (1950).

(20) Purchased from Polarad Electronics Corp., Long Island City, N. Y.

(21) In no case was appreciable titration observed above pH 6, using the DNA samples mentioned in the Experimental section. Furthermore, electrophoresis of D-V DNA in 0.1 ionic strength phosphate buffers of pH 5.99 and pH 6.89 showed identical mobilities (-17×10^{-5}), within the error of the experiment ($\pm 4 \times 10^{-5}$). The increasing mobility above pH 6 reported by A. R. Mathieson and J. V. McLaren, *J. Chem. Soc.*, 164 (1956), for DNA prepared by the method of Gulland, Jordan and Threlfall, is in accord with the high titer of a similarly prepared sample in this (secondary phosphate) region, found by Dr. Elio Polli in our laboratory.

various temperatures, an inflection point occurs at a pH which rises as the temperature rises,[22] although the initial and final portions of the forward curves, and the entire back-curves, remain coincident (Fig. 1). Forward and back titrations to and

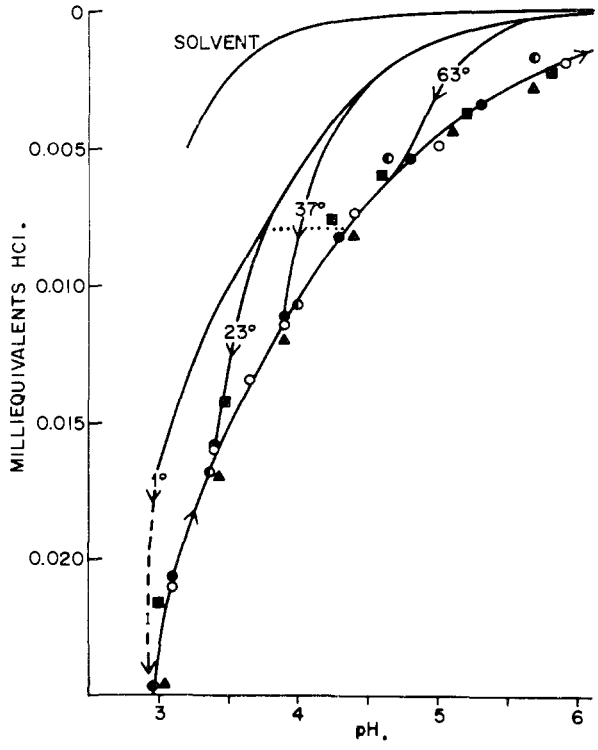

Fig. 1.—Acid titration curves at various temperatures, for 7 ml. of a 1.04 mg./ml. DNA solution of ionic strength 0.017. Solvent curve, at top, is essentially the same at all temperatures. The points indicate the back-titration curves at each temperature: ■, 1°; O, 23°; ●, 37°; △, 63°. The *forward* titration curve at 75° is indicated by O, and coincides with the back-titration curve at lower temperatures. The dashed portion of the forward curve at 1° has been displaced upward for convenience in plotting. The horizontal dotted line refers to the 23° titration, and is discussed in the text.

from different points along the forward curve[6] at any one temperature have established that irreversibility commences in the vicinity of the inflection. That is, before inflection the titration curve is reversible and after inflection it is not. The inflection, then, is caused by the shift of the base pK_a's to higher values, which occurs during denaturation. Since the only temperature-dependent part of the titration curve is the position of the inflection, which reflects a mixture of the pK_a''s of denatured and undenatured groups, these pK_a''s themselves must each be the same at all temperatures; only the point of transition from one to the other changes. The low temperature coefficient of the base pK_a''s has been reported by Cox[17] and also has been found in this Laboratory.

The foregoing results are summarized in Fig. 2, which shows that, for any given ionic strength, the

(22) The possibility that this is a rate effect was investigated and eliminated. Prolonged exposures to various pH's at different temperatures did not change the results.

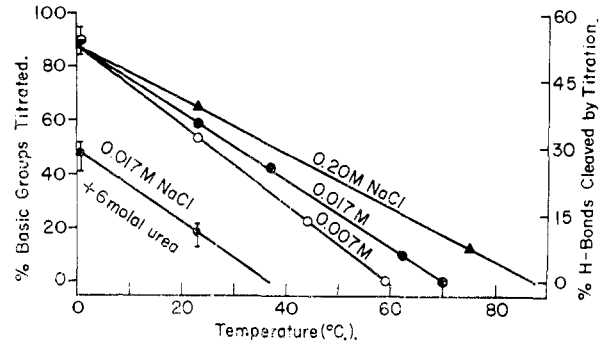

Fig. 2.—Maximum number of basic groups (% of the total) reversibly titratable, as a function of temperature. For each set of conditions, DNA will be denatured above the appropriate curve. Vertical lines show the size of the error for the less accurate points. The ordinate on the right is explained in footnote 23.

total number of bases which may be titrated (*i.e.*, the number of hydrogen (H-) bonds which may be directly cleaved by titration[23]) without causing denaturation is a linear, inverse function of the temperature. That is, fewer equivalents of acid are required to initiate denaturation as the temperature rises. Cox and Peacocke[17] have found, similarly, that denaturation is complete at 35° when less acid has been added than at 25°. A temperature is finally reached (about 70° in 0.017 M NaCl, for example) at which denaturation occurs without titrating any bases, and cannot be prevented by heating in buffers of any pH. In such cases, the original H-bonds of DNA must all[24] be thermally and irreversibly cleaved; at lower temperatures, fewer are thermally broken and therefore some must also be broken by titration in order to initiate denaturation. At each point on the curves in Fig. 2, reversible thermal and titrational cleavage have progressed so far that only the critical number of intact H-bonds remains. A slight increase in the number of bases titrated will result in partial denaturation, *i.e.*, the spontaneous irreversible cleavage of all the H-bonds in certain molecular regions. Since denaturation results in increased pK_a''s, a sudden and complete cleavage of all H-bonds would yield a curve similar to that shown by the horizontal dotted line in Fig. 1 (for 23°). Actually, an inflection is observed, due to the non-random base distribution and heterogeneity of the H-bond strengths in DNA. One may imagine that there are separate regions in a DNA molecule, each of which requires a certain minimum sequence of H-bonds to remain native, and that these regions, being heterogeneous, are denatured one by one as increments of acid are added (over a 0.3 pH range). However, the present data cannot distinguish between this case and one in which each of a collection of different molecules is denatured as a whole.

(23) Figured on the basis that 1 milliequivalent of acid equals 1 milliequivalent of H-bonds cleaved and that there are five H-bonds for every four bases (see ref. 33). Thus, three-fifths of the total number of H-bonds in a molecule may be cleaved directly by acid, assuming the adenine–guanine ratio to be about 1.

(24) Physical measurements have shown (*e.g.*, ref. 5) that the structural order which results from H-bonding disappears during denaturation. If the two strands of the denatured molecule are held together by a small number of residual H-bonds, these are not necessarily of the original type.

When acid denaturation is observed spectrally[25] at 260 mμ, in both dilute and concentrated solution, the usual optical density increase of approximately 40% is found to occur in the same, narrow *p*H range as potentiometric irreversibility and to be the same function of ionic strength and temperature. It can be thus concluded that denaturation is independent of DNA concentration, if the ionic strength is kept constant. The spectral curve at 260 mμ, being much steeper than the potentiometric curve, is not a true titration curve.[8] That is, the optical density does not increase gradually as the bases are ionized; rather, its rise reflects the structural changes that occur during denaturation. Table I is a compilation of potentiometric and spectral data.

TABLE I

*p*H RANGE IN WHICH DENATURATION OF DNA OCCURS

Temp., °C.	Ionic strength	Potentiometric *p*H range[a]	Spectral *p*H range[b]
1	0.007	3.1–2.8	
23	.007	4.0–3.7	4.3–3.6
63	.007	.....[c]	.....[c]
1	.017	<3.3	2.65–2.35
23	.017	3.7–3.4	3.7–3.4
37	.017	3.9–3.6	4.0–3.6
63	.017	>4.6	5.4–5.1
75	.017	.....[c]	.....[c]
1	.20	<2.6	
23	.20	>2.6	2.9–2.6
63	.20		4.2–3.7
75	.20		4.4–4.1

[a] *p*H range for the onset and completion of irreversibility. [b] *p*H range for the optical density increase at 260 mμ. [c] These solutions are thermally denatured, they possess a high extinction coefficient, and potentiometric titration shows that they are denatured.

The effect of salt in shifting the titration curves of DNA has been demonstrated[3,11] and attributed to, among other things, the presence of phosphate fields.[8,5,26] Salt also has another effect: namely, the protection of hydrogen bonds from thermal cleavage.[27] This is demonstrated in Fig. 2 by the curves for different salt concentrations, which show that above 0° the number of reversibly titratable bases increases with the salt concentration. Evidence also has been obtained that chelating agents, such as glycine, exert a protective influence beyond that attributable to their contribution to the ionic strength.[28] Heating experiments carried out in chelating buffers may therefore not be compared quantitatively to those in ordinary salts or phosphate buffers.

(25) Alkali titrations have also been followed spectrally. As the temperature rises, the point of denaturation (optical density increase at 260 mμ) shifts to lower *p*H's and presumably to a smaller number of bases titrated.

(26) Present calculations indicate that the fields permissible in the Watson–Crick model can exert local effects which will account for the large pK_a' shifts observed on varying the salt concentration, and for the differences between polymer and nucleoside pK_a''s. Support for this view has been provided by T. L. Hill, THIS JOURNAL, **78**, 3330 (1956).

(27) See R. Thomas (ref. 9) and P. D. Lawley (ref. 38) regarding optical density increase; Zamenhof, Alexander and Leidy (ref. 4), regarding biological activity; Cox and Peacocke (ref. 3) regarding potentiometric titration; and T. Miyaji and V. E. Price, *Proc. Soc. Exptl. Biol. & Med.*, **75**, 311 (1950), regarding viscosity.

(28) See also ref. 14.

Although 6 *m* urea does not denature DNA at room temperature,[29] it does raise the *p*H at which potentiometric inflection, irreversibility and optical density increase occur at any given temperature. Since urea has no effect on the ionic strength and almost none on the dielectric constant, it does not shift the titration curves. Rather, its effect is like that of a temperature increase: it decreases the number of bases that may be titrated before denaturing (Fig. 2) and thus must increase the number of thermally cleaved H-bonds.

These experiments offer an explanation for some conflicting data in the literature. Although the physical changes accompanying denaturation in DNA have always appeared to be largely irreversible, Reichmann, Bunce and Doty[30] have reported light-scattering data showing that the collapse of the molecule at *p*H 2.6 in 0.2 *M* salt may be reversed by dialyzing back to neutrality in the cold. It seems probable that their entire reversible procedure for lowering and raising the *p*H was carried out in the cold, while a sample at *p*H 2.6 was warmed to room temperature (at which time denaturation, accompanied by irreversible collapse, occurred) before measuring its scattering. We have checked this point by measuring the viscosity, at 1.8 and 22.3°, of DNA solutions in 0.2 *M* NaCl at *p*H 2.6 and *p*H 6.1. The *p*H's were adjusted directly with 1 *N* HCl; no special precautions were taken. The results, in Table II, show that at 1.8° a very small, but completely reversible contraction occurs at *p*H 2.6; but when the solution at *p*H 2.6 is brought to room temperature, the molecule collapses irreversibly. There can be no question of local degradation here. These results agree with other reversibility measurements (*e.g.*, Table I, and the light scattering results of Alexander and Stacey[31]).

TABLE II

SPECIFIC VISCOSITY[a]

Temp., °C.	*p*H 6.1	*p*H 2.6	*p*H 6.1[b]
22.3	0.172	0.000	0.029
1.8	0.170	0.124	0.165

[a] DNA 0.063 mg./ml. in 0.2 *M* NaCl. [b] Reneutralized after ~15 minutes at *p*H 2.6.

In our titration experiments, variation of acid normality and speed of addition had no effect on the results. We feel, in sum, that the possibility of local acid action during addition is remote.

Discussion and Conclusions

Hydrogen bonds in DNA can be cleaved reversibly, either thermally or by titration or by a combination of both, until a certain minimum number of intact H-bonds remains. Thereafter, the remaining bonds cleave spontaneously and irreversibly, region by region or molecule by molecule, over a narrow *p*H range whose width is related to H-bond heterogeneity. The latter process has

(29) At much lower concentrations (0.7 *M*), uridine causes a 40% drop in the specific viscosity of DNA. Cytidine and ribose, at the same concentration, have no effect. This suggests that uridine is a very effective H-bonding agent.

(30) M. E. Reichmann, B. H. Bunce and P. Doty, *J. Polymer Sci.*, **10**, 109 (1953).

(31) P. Alexander and K. A. Stacey, *Biochem. J.*, **60**, 194 (1955).

been called denaturation. The notion that ionization of a base permanently breaks its own and no other hydrogen bonds, thus implying denaturation to be a continuous and non-coöperative process,[32] is untenable. The experiments reported here (see Fig. 2) show that when thermally and titrationally cleaved bonds are both present, each in amounts which alone are reversible, denaturation can result. That is, the effects of heat and acid, when present simultaneously, are additive, and denaturation is apparently the same regardless of the means by which the H-bonds have been broken.

From the titration curves (Fig. 1), it is apparent that only the initial part of the DNA forward titration curve at room temperature reflects the "native" base pK'_a's; if these are to be calculated, one must construct a theoretical curve, coinciding with the 1° forward curve except at the lower end. The guanine region of this curve will not be reversible, thus refuting an old argument for the non-participation of the guanine amino group in H-bonding. Indeed, it is almost certainly H-bonded.[33] The calculation by Jordan, *et al.*,[32] of pK'_a's from the "forward" curve at room temperature is therefore invalid. Furthermore, since both undenatured and denatured pK'_a's are functions of ionic strength, pH experiments in which the salt concentration is inconstant or unspecified are impossible to interpret. In connection with this salt effect, we have found that the pK'_a's are independent of DNA concentration when the counterion contribution to the ionic strength is taken into account.

Since at 1° titration of guanine[34] as well as adenine and cytosine is necessary to bring about denaturation, proton addition to any of the three bases must break H-bonds. One must conclude, on the basis of the Watson–Crick pairing, that the protons add largely to the 1-nitrogens of cytosine and adenine, thereby cleaving the H-bonds at those positions. Since there are no acceptor nitrogens in guanine, the situation is more complex. A mechanism has been proposed[6] involving proton addition to the amino group, followed by enolization and cleavage of the guanine oxygen–cytosine amino H-bond.

It can be further concluded that the mere presence of H-bonds does not account for the fact that the titration curve of undenatured DNA lies in a lower pH region than that of denatured DNA (the back-titration curve). That H-bonds can be cleaved without affecting pK'_a's is proved by the reversibility of the titration curves before denaturation, and by the coincidence of the undenatured portions of the forward curves at various temperatures, in spite of the fact that widely varying proportions of the groups titrated are no longer involved in H-bonds (having been previously thermally cleaved). Apparently the pK'_a increase which occurs during denaturation is a consequence of irreversible configurational changes.

Some light has been shed on the mechanism of thermal H-bond cleavage in undenatured DNA by the experiments reported in the previous section. It is clear from Fig. 2 that progressively fewer groups may be titrated as the temperature increases, before denaturation begins. We interpret this as indicating that more bonds are thermally broken at elevated temperatures, and that before denaturation actually begins there must exist a (temperature-dependent) equilibrium situation involving H-bond cleavage. (The reaction becomes irreversible as soon as the equilibrium constant permits the existence of fewer than the minimum number of H-bonds, N, necessary to maintain the molecule in its undenatured state.) The urea experiments in Fig. 2 lend support to this interpretation. The effect of urea is to lower the temperature at which denaturation occurs (for any given titer). This may be due to an increase in the equilibrium constant for the thermal reaction (intact $\rightleftarrows$ broken H-bonds) and/or an increase in the value of N. Since urea does not alter ionic strength or dielectric constant (to any significant extent), it is quite probable that the environment of the DNA is unchanged and therefore that N is not increased. Thus, urea must break base–base H-bonds to form base–urea H-bonds, more effectively than does water to form base–water H-bonds, *i.e.*, the equilibrium constant is increased by urea. Actually, then, thermal cleavage is the replacement of one type of H-bond by another. Presumably, in the absence of hydrogen-bonding solvent, the temperature required to initiate denaturation would be much higher.[35] The equilibrium for thermal H-bond cleavage, then, depends on the solvent as well as the temperature.

Attempts to estimate average thermodynamic functions for reversible thermal H-bond cleavage from the data in Fig. 2 have failed because of the unknown temperature-dependence of N. There is also an open question as to how many bonds between a given base pair break once one of them has been titrated. This is also a thermal cleavage, but it would weight the (average) equilibrium constant calculated in the presence of acid to a higher value than would obtain at neutrality. Although it has been suggested[17] that titration breaks *all* the H-bonds between the given pair (*e.g.*, guanine–cytosine), this cannot be universally true in view of the aforementioned participation of both cytosine and guanine ionization in denaturation under certain conditions.

Ionic strength very probably also affects the equilibrium constant for thermal cleavage (Fig. 2). The fact that all the salt curves intersect, at a temperature near 0°, suggests that at this point the salt-dependent quantity may disappear. It is known that some intact H-bonds are present at this temperature, since further cleavage by addition of acid will bring about denaturation.[36] It is pos-

(32) D. O. Jordan, A. R. Mathieson and S. Matty, *J. Chem. Soc.*, 154 (1956).

(33) L. Pauling and R. B. Corey, *Arch. Biochem. Biophys.*, **65**, 164 (1956).

(34) The involvement of guanine titration in denaturation has been confirmed by titrating a guanine-rich thymus DNA fraction prepared by the method of C. F. Crampton, R. Lipshitz and E. Chargaff, *J. Biol. Chem.*, **211**, 125 (1954). Denaturation occurred when about the same number of bases had been titrated as in the unfractionated DNA, but at a lower pH.

(35) J. A. Schellman, *Compt. rend. trav. lab., Carlsberg, ser. chim.*, **29**, 230 (1955).

(36) Cox and Peacocke (ref. 17), in the belief that guanine ionization was not involved in denaturation, apparently did not carry out their 0.4° titration to a sufficiently low pH to break all the H-bonds. Therefore they did not reach the completely denatured state at this temperature.

sible, however, that the equilibrium constant for thermal cleavage may be insignificantly small at 0°. This would imply that the equilibrium constant is salt-dependent and that the number of essential H-bonds (N) may not be. Thus the protective effect of salt might be exerted on the surrounding water, which is involved in the thermal equilibrium, possibly by disrupting its semi-crystalline structure in the vicinity of the DNA molecule.[37]

Denaturation is, by definition, an irreversible process. Thus, as has been shown here (Table II) and elsewhere,[5,31] the molecular collapse and the pK'_a shifts which accompany it are permanent. Whenever this appears not to be so, one must suspect that a variable such as temperature has not been controlled, and that denaturation did not actually occur. The optical density rise, however, may appear under some conditions to be reversible, since the extinction coefficient at 260 mμ, which is constant before denaturation, afterward becomes salt, pH and temperature dependent. Therefore optical density cannot validly be used as an indicator for denaturation unless the measurement is carried out at the temperature or pH of the experiment. Cooling to room temperature, or neutralizing, will not reverse the collapse of the molecule, but, as Lawley[38] has shown, it will decrease the optical density rise.

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Denaturation and degradation appear to be separate phenomena, although caused in many cases by the same agent. For example, the formation of apurinic acid at low pH does not proceed to any significant extent[39] under the conditions and durations of the experiments reported here, although denaturation does. A number of investigators[40] have reported a decrease in molecular weight, usually followed by aggregation[40d] or thixotropic[40a-c] behavior, after heat treatment of DNA. The conditions requisite for this effect need not be the same as those which bring about denaturation. There are indications that, at least in lower salt concentrations, denaturation occurs under milder acid and heating conditions than degradation.[40d] Our observations suggest that denaturation is a rapid reaction, while degradation by either acid or heat is undoubtedly slower.

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Near Infrared Spectra of Compounds with Two Peptide Bonds and the Configuration of a Polypeptide Chain. VI. Further Evidence of the Internal Hydrogen Bonding and an Estimation of its Energy

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Near infrared spectra of acetylsarcosine N-methylamide and acetyl-N-methyl-DL-norleucine N-methylamide have been measured in carbon tetrachloride and chloroform solutions. Even at high dilutions both the free and bonded NH vibrations have been observed and have been explained as arising from the extended and folded molecular forms, respectively. The dependence of the intensity of the two NH bands upon temperature has been determined and the energy difference between the extended and folded forms have been calculated.

Introduction

In this series of researches we have studied the structure of molecules of the type

$$CH_3CONHCHRCONHR' \quad I$$

which can be considered as a structure unit of a polypeptide chain.[1] From the measurement of the NH absorption bands we have concluded that such molecules exist in both the extended and folded forms shown in Fig. 1. They show the free NH absorption at about 3460 cm.$^{-1}$ and the bonded NH absorption at about 3360 cm.$^{-1}$ in dilute carbon tetrachloride solution and in chloroform solution. In contrast to the band due to the *intermolecular* hydrogen bond, the 3360 cm.$^{-1}$ band shows no dependence on concentration in a dilute solution which shows the band arises from the *intramolecular* hydrogen bond[2] of the folded form of Fig. 1A. This is compatible with the result of our measurement on acetylamino acid ester

$$CH_3CONHCHRCOOR'' \quad II$$

for which we found no absorption due to the intramolecular hydrogen bond.[3] It would be interesting to see whether we can observe the bonded NH absorption at high dilution for a molecule of the type

$$\begin{array}{l} CH_3CONCHRCONHR' \\ \quad\quad\quad\quad | \\ \quad\quad\quad\quad CH_3 \end{array} \quad III$$

with only one NH group in the molecule, the hydrogen atom of the other NH group being replaced by a methyl group.

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