830. *Electrometric Titration of the Sodium Salts of Deoxyribonucleic Acids. Part VI.* The Forward-titration Curve.*

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Dissociation curves have been obtained at 25° in 0·05M-sodium chloride for a neutral solution of herring-sperm sodium deoxyribonucleate titrated with acid to a series of pH values higher than that corresponding to complete denaturation and then back-titrated to neutrality. Thence is derived the relation between the proportion (β) of the original double-helical structure which became disordered and the degree of ionisation (γ) of the initially hydrogen-bonded amino-groups. Below a critical value of γ this relation follows closely that predicted on the assumption that co-operative ionisation of two adjacent hydrogen-bonded groups is the necessary condition for the disordering of any part of the original structure. At the critical value of γ (*viz.*, $\gamma = 0{\cdot}75$, when $\beta = 0{\cdot}75$) there is apparently a sudden breakdown of the remaining hydrogen-bonded structure into a completely disordered form in which any hydrogen bonds are randomly disposed and are not the specifically directed bonds of the double helix. These conclusions are confirmed by the ultraviolet absorption of the nucleate after various titration cycles.

The mechanism suggested by these results has been used to analyse earlier observations on the effect of changes in temperature and in ionic strength on the relation between the forward- and the back-titration curves both above and below pH 7. The energy relations between the various forms of the deoxyribonucleate molecule and related problems are also discussed.

The hydrogen bonds cross-linking the two helical polynucleotide chains of sodium nucleate may be irreversibly ruptured by heat,[1] ionising radiation,[2] acid, or alkali,[3,4] and the proportion of hydrogen bonds destroyed by these agents may be computed [1] from the relative positions of the forward- and the back-titration curves obtained on subsequent titration. The irreversible rupture of hydrogen bonds (" denaturation ") during forward-titration from neutrality with acid or alkali has not hitherto been examined closely, although certain features of the effects of ionic strength [5] and of temperature [6] on the forward-titration curves have suggested that it may not always be necessary to ionise all the hydrogen-bonded amino-groups in order to attain complete denaturation and that the ease of denaturation by acid increases with temperature. Two limiting forms of behaviour have also been observed: complete ionisation without denaturation [6,7] at low temperature and, conversely, complete denaturation [1] by heat without change of pH.

We now report a more detailed study of acid denaturation made by first lowering the pH to various points intermediate between neutrality and the pH of complete denaturation and then back-titrating the solution with alkali. The relative positions of the curves obtained in this way must depend on the course of denaturation as a function of pH and should be informative about the mechanism of acid denaturation. Parallel ultraviolet-absorption measurements have also been made since the intact double-helical structure appears to have an especially low extinction which increases irreversibly on denaturation.[8,9]

Experimental

The preparation and characteristics of the herring-sperm sodium deoxyribonucleate used have been given elsewhere.[5]

* Part V, *J.*, 1957, 4724.

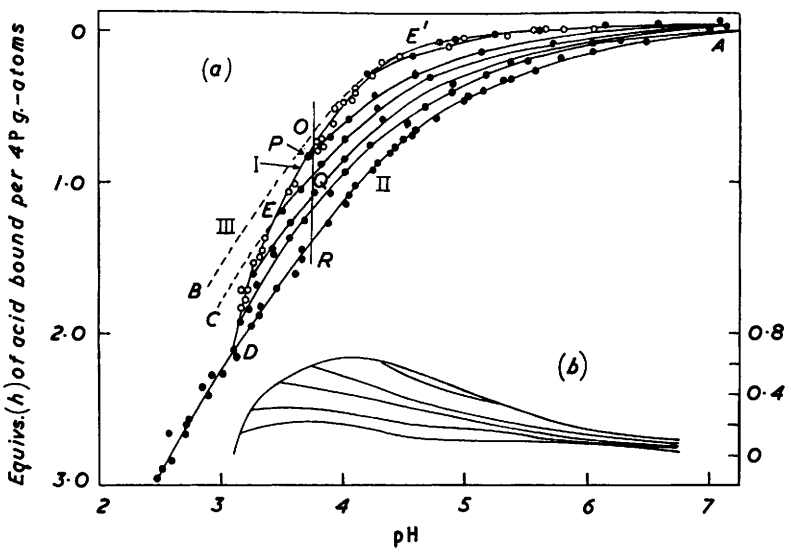
[1] Cox and Peacocke, *J.*, 1956, 2646.
[2] Cox, Overend, Peacocke, and Wilson, *Nature*, 1955, **176**, 919.
[3] Gulland, Jordan, and Taylor, *J.*, 1947, 1131.
[4] Lee and Peacocke, *J.*, 1951, 3361.
[5] Cox and Peacocke, *J.*, 1956, 2499.
[6] *Idem*, *J.*, 1957, 4724.
[7] Preston and Peacocke, unpublished work; Peacocke, *Chem. Soc. Special Publ.*, No. 8, 1957, p. 161.
[8] Laland, Lee, Overend, and Peacocke, *Biochim. Biophys. Acta*, 1954, **14**, 356.
[9] Thomas, *ibid.*, p. 231.

Solutions of sodium nucleate (0·2% in 0·05M-sodium chloride) were titrated electrometrically in cells with liquid junctions by a continuous method, as described previously.[5] 0·1N-Hydrochloric acid was added in 0·0002 ml. portions to solutions well stirred by a stream of nitrogen, until the required pH (between 3·1 and 7) was attained. Alkali was then added in a similar manner to pH 10.

Titration curves are shown as the corrected number (h) of equivalents of acid (*i.e.*, hydrogen ions) bound by an amount of the nucleate containing 4 P g.-atoms as a function of pH on a standard scale [10] on which 0·05M-potassium hydrogen phthalate has a pH of $4{\cdot}00_5$ at 25°.

The ultraviolet absorption values of the various acid-treated nucleate solutions (pH 7·5, in 0·05M-sodium chloride), after dilution with 0·05M-sodium chloride and centrifugation (45 min., 5000 r.p.m.), were measured against 0·05M-sodium chloride with a Unicam spectrophotometer. The extinction coefficients of several independent dilutions of the same stock solution agreed within 1%.

FIG. 1. (*a*) *Titration curves of herring-sperm sodium deoxyribonucleate at* 25° *in* 0·05M-*sodium chloride solution: the effect of graded acid treatment.*



I, *Irreversible forward-titration curve with acid* (*APED*).
II, *Reversible back-titration curve with alkali from D or lower* pH (*DRA*).
III, *Reversible titration curve of the intact* DNA *structure* (*AE'OB*).
EQA, Reversible back-titration curve with alkali from E (*similarly for the other intermediate curves*).
(*b*) (*Lower figure*), *Difference curves. Ordinate scale* (*lower right*) *represents the acid bound along curve DRA* minus *the acid bound along the various intermediate curves, such as AQED.*

Results.—The continuous titration curves at 25° in 0·05M-sodium chloride are shown in Fig. 1. Curve I is the usual irreversible forward-titration curve for the sodium nucleate and points obtained by many independent titrations all fall accurately on it; curve II is the reversible back-titration curve and there is substantial evidence [1] that it represents the dissociation curve of non-hydrogen-bonded nucleate. The curves intermediate between I and II were obtained by back-titration from various pH's higher than the point (*D*) of intersection of I and II. The forward-titration curve was reversible between pH 4·55 and pH 7·00, and the curve obtained on back-titration from pH 4·20 was little different from curve I. This implies that at 25° all of the first 10% and most of the first 18% of the hydrogen-bonded amino-groups that are titratable gain and lose protons reversibly.

At pH's less than 4·20 the difference between the forward- and the back-titration curves is marked and the curves shown between I and II (Fig. 1) must correspond to mixtures of hydrogen-bonded and non-hydrogen-bonded nucleate.[1,11] The steep gradient of the forward-titration curve over the range pH 4·25—3·10 particularly between pH 3·10 and 3·15 shows

[10] British Standard 1647: 1950.
[11] Cox and Peacocke, *J. Polymer Sci.*, 1957, **23**, 765.

that larger irreversible changes in hydrogen-bonding in the nucleate are brought about by very small changes in pH in this region.

The curves intermediate between I and II were shown to be reversible in the following way. Nucleate was titrated with acid to pH 3·50 (*E*) and the curve (*EQA*) was followed on back-titration with alkali to neutrality (*A*). The curve for the subsequent re-titration with acid from *A* was identical with *EQA* over the range pH 7·00—3·50 down to *E* and followed I between this point and pH 3·10 (*D*). Curve II was followed on back-titration with alkali from pH 3·10 (*DRA*). Thus the partly denatured nucleate, whose pH had previously been reduced to *E* and then increased to *A*, had the forward-titration curve *AQED* with acid and on back-titration with alkali from *D* gave the usual curve II. Fig. 1(*b*) is a plot of the difference in acid bound between *AQED* and *DRA* (II) for varying positions of the reversal point *E*, and thus for nucleate samples denatured by acid to various graded extents.

Although the initial titration with acid at 25° resulted in irreversible changes in the nucleate the pH was always stable for at least 30 min. after the addition of acid, and the forward-titration curves were accurately reproducible. Thus any further denaturation that may have followed the first rapid opening up of the hydrogen bonds must have proceeded too slowly to affect the titration behaviour of the nucleate partly denatured with acid. Denaturation by alkali has similarly been shown [12] to take place instantaneously since the pH during forward-titration with alkali has been reported as stable for at least 18 hr.

The ultraviolet absorption at λ 200—300 mμ of nucleate solutions which had undergone the various titration cycles was measured at pH 7·5 in 0·05M-sodium chloride. An increase in

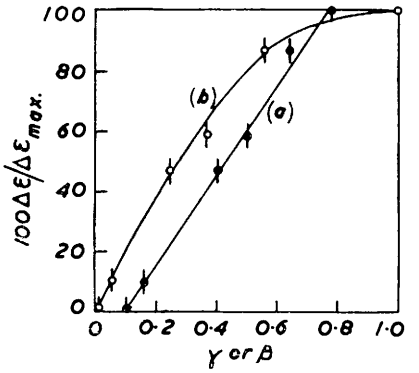


FIG. 2. *The effect of graded acid treatment at 25° on the ultraviolet absorption of sodium deoxyribonucleate at λ 260 mμ.*

Ordinate: Increase in absorption (Δε) *represented as a percentage of the maximum increase* ($\Delta\varepsilon_{max.}$) *attained. Abscissa: For curve* (*a*), *the fraction* (γ) *of groups originally involved in hydrogen bonds which were ionised at reversal point* (*P*, *E*, *etc.*, Fig. 1) *of the titration cycle. For curve* (*b*), *the fraction* (β) *of the original hydrogen bonds which have been permanently ruptured by the titration cycle* (*for calculation, see text*).

absorption compared with the original nucleate was observed in all cases where acid treatment resulted in a difference between the forward- and the back-titration curves, *i.e.*, when denaturation occurred. This increase, represented as a percentage of the maximum attainable, is shown in Fig. 2(*a*) as a function of the fraction (γ) of the groups originally involved in hydrogen bridges which were *ionised* at the reversal point (*P*,*E*, etc.) of the titration cycle that each nucleate solution had previously undergone. At $\gamma > 0{\cdot}1$ the relation is linear and the maximum increase in absorption (14% of the original absorption at λ 260 mμ) was attained when $\gamma = 0{\cdot}75$; from all previous evidence,[1,8] this maximum corresponds to complete breakdown of the hydrogen-bonded structure. The dependence of the increase in ultraviolet absorption on the fraction (β) of the original specific hydrogen bonds which have been *permanently ruptured* is also shown, in Fig. 2(*b*), and is discussed below, where it is shown that β and γ are not necessarily equal since ionisation can occur without the hydrogen bonds' being permanently broken.

DISCUSSION

There is now considerable evidence that the nucleate ion retains the double-helical configuration of the solid state when dissolved in neutral salt solutions and that, at 25°, this configuration may be lost on treatment with acid, *e.g.*, to pH 3·10 in 0·05M-sodium chloride. The " anomalous " titration curve of the nucleate may then be attributed to the irreversible rupture of the complementary hydrogen bonds which bridge the two helices and to the loss of the double-helical structure, a process designated as " denaturation " in this discussion. The results reported above show that the pH may be lowered

[12] Shack and Thompsett, *J. Biol. Chem.*, 1952, **197**, 17.

to 4·5 (E') without the occurrence of any irreversible change. The number of groups ionised at this pH corresponds to about 10% of those amino-groups which were originally involved in hydrogen bridges at neutrality. Thus above pH 4·5 the ionisation of an amino-group and the consequent disappearance of the hydrogen bridge is not sufficient to cause a permanent change in configuration. The reversible dissociations occurring over this pH range may be represented as:

$$\underset{(a)}{\{\text{Helix}\}\text{–}NH_3^+ + (\text{–}NH\cdot CO\text{–})\{\text{Helix}\}} \rightleftharpoons \underset{(b)}{\{\text{Helix}\}\text{–}NH_2 \ldots (\text{–}NH\cdot CO\text{–})\{\text{Helix}\}} + H^+ \quad \ldots \quad (1)$$

where $\text{–}NH_3^+$ and $\text{–}NH_2$ are the charged and the uncharged form of the 1 : 6-amino-system capable of being hydrogen-bonded (adenine and cytosine 6-amino-groups [13] and, possibly,[14] the guanine 2-amino-group), (–NH·CO–) is the system to which these groups are hydrogen-bonded in the double helix (the 1 : 6–NH·CO– systems of thymine and guanine [13] and, possibly,[14] the cytosine 2-oxo-group); the terms enclosed in braces represent the configuration of the polynucleotide chains and the dotted line represents a hydrogen bond * of the complementary type.[13] At lower temperatures (below −0·4°) the dissociations appear to be of this type over the whole pH range and the double helical configuration is presumed to be retained.[7] The reversible forward-titration curve of nucleate in the double-helical form at −0·4° has recently been observed directly [7] and was first inferred [6] from the curves at +0·4°; from it the reversible forward-titration curve at 25° may be deduced (III, $AE'OB$, of Fig. 1) and is, in fact, almost identical with it because of the low heats of dissociation of the 6-amino-groups of adenine and cytosine.[6]

The appearance and growth of the titration anomaly at 25°, as the pH at the reversal point became more acid than 4·5, indicates the onset and increasing extent of denaturation, which was complete at pH 3·10, all the 6-amino-groups of adenine and cytosine and some of the guanine 2-amino-groups being then ionised. On back-titration with alkali from pH 3·10 or lower, dissociation of the denatured nucleate (curve II) was reversible and may be represented as:

$$\underset{(c)}{\left\{\begin{matrix}\text{Denatured}\\ \text{chain}\end{matrix}\right\}\text{–}NH_3^+ + H_2O} \rightleftharpoons \underset{(d)}{\left\{\begin{matrix}\text{Denatured}\\ \text{chain}\end{matrix}\right\}\text{–}NH_2 \ldots (H_2O)} + H^+ \quad \ldots \quad (2)$$

The notation is the same as for (1) except that the dotted lines now represent hydrogen bonds of a non-specific type involving solvent molecules. The dissociation curve for a mixture of the two forms should be intermediate between I and II at a position determined by its composition. If R and O are, respectively, points at a given pH on the reversible titration curves (II, III) shown in Fig. 1 for denatured and undenatured nucleate and if Q is a point on the (reversible) curve for a mixture containing a fraction β of the nucleate in the denatured form (measured as nucleotide units), then, by a previous argument,[1] $\beta = OQ/OR$. Values of β deduced in this way from the intermediate back-titration curves † (*e.g.*, EQA of Fig. 1) are given in Table 1 and, over the pH range in which it can be calculated, β was reasonably independent of pH. Thus β may be obtained for any point P on the usual forward-titration curve from a *single* calculation of OP/OR for that point, and hence the variation of β with the number of groups ionized over the whole of the forward-titration can be deduced.

A point on the forward-titration curve, which corresponds to an extent of denaturation β, must also be a point on the titration curve for (1 − β) mole of undenatured and β mole of denatured sodium nucleate. Thus the point E at pH 3·50 lies on the curve $CEQA$

* Here, and in what follows, the term hydrogen " bond " or " bridge " in the singular will refer, unless otherwise indicated, to the two (or three) such bonds regarded as linking a pair of bases, one in each helical polynucleotide chain.

† The reversibility of these curves shows that there is no denaturation as a result of high local concentrations of acid or alkali when these reagents are added.

[13] Watson and Crick, *Nature*, 1953, **171**, 737.

[14] Pauling and Corey, *Arch. Biochem. Biophys.*, 1956, **65**, 164.

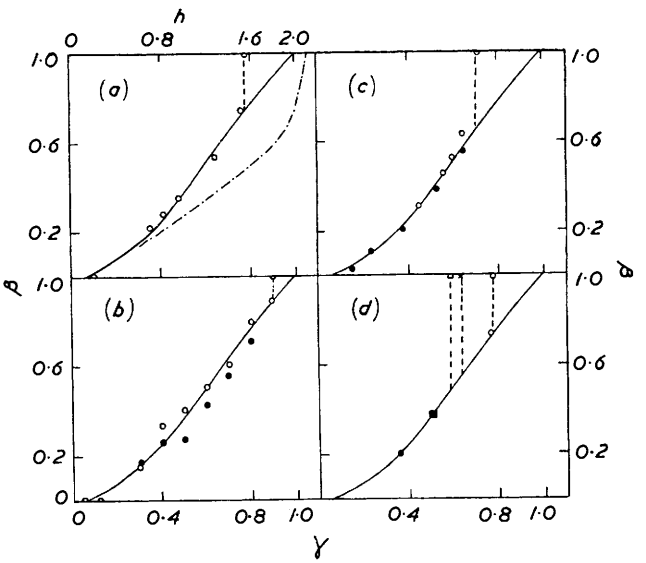
TABLE 1. *The extent of denaturation* (β) *deduced at various points on the intermediate back-titration curves of Fig.* 1.

Back-titration curves from the reversal points		β * at points on these curves								Average β
h	pH	pH: 3·25	3·50	3·75	4·00	4·25	4·50	4·75	5·00	
0·84	3·75	—	—	0·17	0·20	0·22	0·22	0·22	0·25	0·22
1·20	3·48	—	0·27	0·34	0·34	0·38	0·38	0·36	0·38	0·35
1·62	3·25	0·55	0·49	0·49	0·51	0·50	0·58	0·56	0·62	0·53
1·94	3·15	0·70	0·65	0·68	0·72	0·74	0·80	0·76	0·71	0·73

* *E.g.*, at the point *Q* on *EQA* of Fig. 1, $\beta = OQ/OR$ (see main text).

(Fig. 1) for which $\beta = 0{\cdot}35$. Only the portion *EQA* of this curve is experimentally accessible, since when the pH is decreased by a small amount (δpH) from pH 3·50 the

FIG. 3. *The dependence of the fraction* (β) *of hydrogen bonds permanently ruptured on the fraction* (γ) *of 6-amino* (*or* 1 : 6-NH·CO–)*-groups ionised during titration. Ordinate,* β; *lower abscissae,* γ. *In* (*a*)—(*d*) *the full line is the theoretical curve for* β–γ *on the assumption that* $n = 2$ *in equation* (3) (*see text*).



(*a*) ○, *Points derived for* β–γ *from the forward-titration curve with acid at* 25° *in* 0·05M-NaCl (*APED*, Fig. 1).
— · — · — *curve of* β *against the number* (*h*) *of protons bound per* 4P *g.-atoms* (*abscissa at the top of the diagram*).

(*b*) β *as a function of the fraction of* 1 : 6-NH–CO– *groups ionised on forward-titration with alkali* [5] *at* 25°.
○, *In* 0·05M-NaCl; ●, *in* 0·50M-NaCl.

(*c*) *The effect of temperature* [6] *on denaturation occurring during titration with acid in the presence of* 0·05M-NaCl.
○, *Deduced from titration at* 35°; ●, *deduced from titration at* 0·4° (*precipitation occurred at* $\gamma = 0{\cdot}65$).

(*d*) *The effect of ionic strength* [5] *on denaturation during titration with acid at* 25°; *for simplicity only the values of* β *when* $\gamma = 1$ *are shown except at high ionic strengths where precipitation intervened before denaturation was complete.*
□, *Titration in the absence of added salt. Titration at ionic strengths of*: × 0·02; ○ 0·05; ■ 0·15; ● 0·50.
At the last two ionic strengths the nucleate was precipitated from solution at values of γ *higher than those indicated.*

fraction of denatured regions immediately increases to (β + δβ) and it is the curve for a mixture of denatured and undenatured nucleate in the ratio (β + δβ) to [1 − (β + δβ)] which is then followed on back-titration from pH (3·50 − δpH). The forward-titration curve therefore represents a transition from the curve for $\beta = 0$ to that for $\beta = 1$ by way of a continuous series of curves of the type *CEQA*, each representing a more denatured

molecule than the previous one until the double-helical structure is completely destroyed ($\beta = 1$). This transition is shown in Fig. 3(*a*) where β is given as a function of h, which is proportional to the number of hydrogen ions bound. However, h is compounded both of the ionisations of groups originally hydrogen-bonded (6-amino-groups) and of the protons bound by groups not originally hydrogen-bonded, such as primary phosphate groups and, possibly, the guanine 2-amino-groups.* At various pH's the contribution to h of these last two groups was estimated from their pK'_a values and from the titration curves of reduced apurinic acid,[15] so that the fraction (γ) of 6-amino-groups ionised at a given pH was derived. In Fig. 3(*a*) the fraction (β) of hydrogen bonds permanently destroyed is also shown as a function of γ: the curves indicate that the first few ionisations ($\gamma < 0{\cdot}1$) had little effect on the double-helical configuration but that denaturation increased rapidly as the hydrogen-bonded groups became charged until 75% of them were ionised. At this critical point ($\gamma = \gamma_c$) the remaining hydrogen bonds were broken without further ionisation.

This behaviour is consistent with the idea [1,11,16] that co-operative rupture of a number of hydrogen bonds is necessary to ensure permanent denaturation. Since the hydrogen bonds succeed each other regularly along the axis of the two helices, it is reasonable to suppose that a necessary condition for a region of the molecule to be denatured is that a minimum number (n) of hydrogen bonds in sequence must all be ruptured at one time. The number n represents the minimum length of polynucleotide chain which can sufficiently change its configuration by rotation about the covalent bonds of the main chain so that the chance of the helical form's ever being regained is infinitesimal.

It has previously been suggested that the acid-denaturation may be explained in these terms and a relation between n, β, and γ has been derived in terms of a statistical model which assumes a random distribution of protons and that all breaks are equally probable.[11] On this model, the fraction (β) of all hydrogen bonds permanently lost, which is the fraction of such bonds occurring as sequences of length n or more, is: [11]

$$\beta = \gamma^n[n + \gamma(1 - n)] \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

where γ is the fraction of hydrogen bonds broken by random ionisation, *i.e.*, it is the probability at that instant that the linkage between a pair of bases will be severed. The values of β calculated from equation (3) with $n = 2$ are in excellent agreement with those found experimentally over the range $\gamma = 0 - 0{\cdot}75$ [Fig. 3(*a*)]. If the guanine 2-amino-group is hydrogen-bonded to the cytosine 2-oxo-group, as Pauling and Corey have suggested,[14] and stays so bonded until the group is ionised, then the value of γ for a given β will be not more than 10% higher than is indicated by the open circles of Fig. 3(*a*). The value of n would then be a little larger than 2 although comparison of the curve of β as a function of the total number of amino-groups ionised with the family of β–γ curves [11] for various n shows that $n \not> 3{\cdot}0$. However, as has been pointed out elsewhere,[17] it is improbable that a single hydrogen bond on the guanine 2-amino-group would continue to link it with cytosine once the two hydrogen bonds at the respective 1 : 6-positions had disappeared by ionisation of the cytosine 6-amino-group at low h values ($0 - 0{\cdot}5$). Thus, on the present evidence, it appears more reasonable to assume that the guanine 2-amino-group is no longer hydrogen-bonded when the pH is low enough for it to ionise ($h \gg 0{\cdot}1$), whatever its state in the original double-helix may have been. This would be consistent with the observed [5] coincidence of the back-titration curve from pH 2·6 with that from pH 12, at which pH only the hydrogen bonds to the 1 : 6-position of guanine can be directly ruptured by ionisation. A value of 2 for n therefore seems the most likely in the

* Or, equivalently, if these groups are hydrogen-bonded in the original nucleate, they have lost their hydrogen bonds as a result of the rupture of hydrogen bonds at the guanine 1 : 6-position through ionisation of the cytosine 6-amino-group.

[15] Hurlen, Laland, Cox, and Peacocke, *Acta Chem. Scand.*, 1956, **10**, 793.
[16] Doty, Proc. 3rd Congr. Biochem., Brussels, Academic Press, New York, 1955, p. 135.
[17] Peacocke, *Chem. Soc. Special Publ.*, No. 8, p. 139.

present instance and is supported by the agreement obtained under other conditions of temperature and ionic strength (see below). The most important departure from the curve of equation (3) with $n = 2$ is the sudden transition from $\beta = 0{\cdot}75$ to 1·0 at a critical value, $\gamma_c = 0{\cdot}75$. It thus appears that when only 25% of the total number of hydrogen bonds are left the structure becomes unstable and the remaining bonds and helical regions become rapidly disorganised to the completely denatured form. This type of process is sometimes described as a " zipper-like " action since the 25% of the groups which are suddenly released from hydrogen bonds are not at this point ionised: they are ruptured apparently because the entire structure suddenly becomes unstable at this point.

Cavalieri and Rosenberg [18] have put forward a different view of the process of acid-denaturation which attributes it mainly to the ionisation of the guanine 2-amino-groups. This was originally propounded [18b] partly to explain the supposed difference between the reversible curves obtained on back-titration from pH 2·6 and pH 12, but since these have now been shown [5] to be coincident this particular argument cannot apply. This view was also based [18b] on their observation that the forward-titration curve was reversible up to the very steep part, " the point of inflection," where nearly all the cytosine and about half of the adenine 6-amino-groups were considered [18b] to be ionised. Thereafter it became irreversible. This contrasts with the observations recorded in Fig. 1, which shows reversibility only in the first 10—20% of these ionisations. Unfortunately these authors have not published detailed curves and points, so that the necessary detailed comparison between the two sets of observations cannot yet be made. It seems difficult, on their view, to explain the observations at 35° (ref. 6) and at 37° and 63° (ref. 18*a*) which show that the point (*D*) of coincidence of the forward- and the back-titration curves moves with increasing temperature to higher pH and lower h values, where few ionisations of the guanine 2-amino-group can be occurring. (Similar remarks apply to titrations at 25° in the absence of salt.) Their explanation of this observation then has to be based on prior denaturation by heat which is unlikely at these temperatures (see below). For these reasons, the acid-denaturation phenomenon is here attributed to the ionisation of all amino-groups originally involved in hydrogen bonds and not especially to that of the guanine 2-amino-group.

The changes in the ultraviolet absorption of neutral nucleate solutions (25°; ionic strength = 0·05) after titration cycles to various pH values and back are shown in Fig. 2(*b*) as a function of β, deduced as already described. The curve is slightly concave to the β axis and this is not surprising since the cytosine 6-amino-groups are presumably ionised before the adenine 6-amino-groups as the pH is lowered and rupture of these two different types of group may lead to different changes in absorption. The maximum increase in absorption is attained at $\beta \sim 0{\cdot}7$—0·8, rather before the point of complete denaturation ($\beta = 1$). This suggests that when the critical extent of denaturation ($\beta = 0{\cdot}75$) and degree of ionisation ($\gamma_c = 0{\cdot}75$) are reached the molecules are already sufficiently disorganised to have an ultraviolet absorption very little different from that of the completely denatured state at $\beta = 1$.

The transition from $\beta = 0$ to $\beta = 1$ with ionisation also occurs on treatment of nucleate with alkali since the same reversible back-titration curve is ultimately obtained as for acid-treatment.[5] Calculations on the more limited data available on the forward titration with alkali show [Fig. 3(*b*)] that also in this case complete denaturation ($\beta = 1$) occurs before ionisation of all the hydrogen-bonded groups (*viz.*, at $\gamma_c = 0{\cdot}9$). Up to this point, the plot of β against γ, which now represents the $^-NH{\cdot}CO^-$ ionisation, again follows the curve of equation (3) with $n = 2$, although the points are less reliable because of their more indirect derivation.

In previous publications the effects of changes in temperature [6] and ionic strength [5]

[18] Cavalieri and Rosenberg, (*a*) *J. Amer. Chem. Soc.*, 1957, **79**, 5352; (*b*) *Biochim. Biophys. Acta*, 1956, **21**, 202.

on the titration curves of the nucleate were reported. The effects on the forward-titration curves may now be examined with reference to the preceding analysis.

Effect of Temperature.—The effect on sodium deoxyribonucleate of treatment with acid to low pH varies with temperature: at −0·75° all groups are ionised reversibly and there is no denaturation; [7] at 0·4° about 40% denaturation occurs; [6] and at 25° and higher temperatures the maximum degree of denaturation [6] is attained. Heating at pH values close to neutrality causes complete denaturation only at temperatures of 75° or higher [1,16] (according to the sample). Below this critical temperature no denaturation has been observed, which renders improbable Cavalieri and Rosenberg's view [18a] that the displacement [6,18a] of the point D to higher pH values with increasing temperatures in the range up to 63° can be explained by rupture of hydrogen bonds by heat *before* titration. An alternative interpretation of this displacement of the point (D) of coincidence of forward- and back-titration curves is developed below after analysis of the forward-titration curves.

The fraction (γ) of groups, originally hydrogen-bonded, which are ionised at various points on the forward-titration curves at 0·4° and 35° may be computed as for the 25° curves: the pK'_a of the primary phosphoryl groups and the 2-amino-groups of guanine at 0·40° and 35° were estimated from the 25° data by assuming [6] apparent heats of dissociation of zero and 4 kcal./mole, respectively. Values of β were computed for the same points on these curves by the methods described above and are plotted as a function of γ in Fig. 3(*c*). The points again lie on the curve of equation (3) with $n = 2$ and are therefore consistent with co-operative rupture of two adjacent hydrogen bonds. As at 25°, when γ exceeds a critical value (*viz.*, 0·7) at 35° the remaining specific hydrogen bonds and helical regions suddenly break down and all the groups involved change to a state in which their dissociation constants are those of the denatured configuration. This critical value (γ_c) decreases with increasing temperature [cf. Figs. 3(*a*), 3(*c*)] and above a certain temperature (*ca.* 75° for this sample) the molecule can be completely denatured even at neutral pH. The increasing instability of the double-helical structure with rising temperature is therefore manifested in a lowering of γ_c and this appears to be sufficient explanation of the upward displacement of the point D at higher temperatures. At 0·4° γ_c was never attained [1,11] and this is attributed to the *increased* contribution to the observed h of the 2-amino-group of guanine at low temperatures, owing to differences in the temperature coefficients of the pK'_a's of the various amino-groups. (This is further evidence against any special rôle of the guanine 2-amino-group in initiating acid denaturation).

TABLE 2. *Free-energy relations in the dissociations in the forward- and the back-titration curves.*

Ionic strength (μ)	0	0·02	0·05	0·15	0·50
Acid branch ($h>0$)					
† $(\Delta pH)_{h=0}$	0·4	0·8	1·4	1·7	1·7
$(\Delta G_2^\circ - \Delta G_1^\circ)$ (kcal./mole)	0·5	1·1	1·9	2·3	2·3
Alkaline branch ($h<0$)					
†$(\Delta pH)_{h=0}$	—	−1·0	−1·1	−1·2	−1·2
$(\Delta G_5^\circ - \Delta G_4^\circ)$ (kcal./mole)	—	−1·4	−1·5	−1·6	−1·6

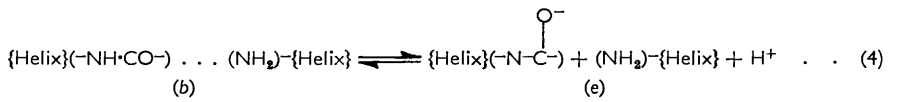
† $(\Delta pH)_{h=0}$ = limiting value of ΔpH as $h \longrightarrow 0$, where $(\Delta pH)_h = (pH_{back} - pH_{forward})_h$ at a given h and is obtained from the 25° curves of Figs. 1—4 (ref. 5).

Effect of Ionic Strength (μ).—It was earlier observed [5] that, at given values of h, both the difference in pH between the forward- and the back-titration curves and the value of h at the point (D of Fig. 1) where these curves meet increase as the concentration of sodium chloride increases. At 25°, when $0 < h < 0{\cdot}3$, and both the forward- and the back-titration curves are reversible (see above), the difference in pH between them at a given h is the positive quantity $(\Delta pH)_h = (pH_{back} - pH_{forward})_h = (pH_2 - pH_1)_h = (pK'_2 - pK'_1)$, where subscripts 1 and 3 refer to the reversible dissociations of the helical and the denatured form, as in equations 1 and 2 and curves III and II (Fig. 1), respectively;

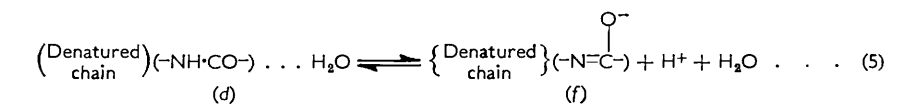
K'_1 and K'_2 are the apparent acidic dissociation constants. Since the corresponding apparent standard free energies of dissociation, $\Delta G°$, are given by $\Delta G° = \boldsymbol{R}T \ln K' = 2{\cdot}303\boldsymbol{R}T \cdot pK'$, it follows that $(\Delta pH)_h = (\Delta G_2° - \Delta G_1°)/2{\cdot}303\,\boldsymbol{R}T$. Values of (ΔpH) and of $(\Delta G_2° - \Delta G_1°)$ as h approaches zero from positive values have been deduced from the curves previously obtained [5] at various ionic strengths (Table 2); the factors contributing to this free-energy difference are discussed in the Appendix.

Plots of β against γ [Fig. 3(*d*)] were derived by the methods outlined above from the forward-titration curves with acid at 25° for the reversal points at different ionic strengths [5] and proved to be similar to those of Figs. 3(*a*) and 3(*c*); a similar mechanism apparently applies with n again equal to 2. The values of γ_c at 25° are calculated to be 0·58 in the absence of added salt and 0·69 at $\mu = 0{\cdot}02$ (cf. $\gamma_c = 0{\cdot}75$ at $\mu = 0{\cdot}05$) (complete denaturation was not attained in forward titration at ionic strengths of 0·15 and 0·50 because of precipitation of the nucleate [4]).

For the alkaline branches of the titration curves ($h < 0$), in which hydrogen ions are being *removed* during the forward titration, $(\Delta pH)_h$ may also be obtained and is here a negative quantity equal to $(\Delta G_5° - \Delta G_4°)/2{\cdot}303\,\boldsymbol{R}T$. The quantities $\Delta G_4°$ and $\Delta G_5°$ are the apparent standard free energies of dissociation of the helical and the denatured form when involved in the following equilibria:



(4)



(5)

Here (–NH·CO–) and (–N=C–) represent the un-ionised and the ionised systems at the 1 : 6-positions of thymine and guanine, and –NH_2 represents the amino-group to which these systems are hydrogen-bonded in the double helix; other symbols are as in equations (1) and (2). [Note that because the complementary hydrogen bonds link the two types of dissociable group, forms (*b*) and (*d*) of equations (1) and (2) also appear in equations (5) and (6). The free-energy differences in the alkali and the acid branch of the curves are compared in the Appendix.] The values for $(\Delta pH)_h$ and $(\Delta G_5° - \Delta G_4°)$ are given in Table 2. In summary, it may be said that the critical degree of ionisation (γ_c) when the partly denatured molecule suddenly breaks down decreases on increase of temperature and on decrease of ionic stength: and that, apart from the direct effects of temperature and ionic strength on the pK'_a of the ionising groups, it is changes in γ_c which appear to be chiefly responsible for the altered shapes of the forward-titration curves relative to the back-titration curves.

The energy relations of the various forms (*a*)—(*d*) that are involved in titration with acid present an interesting problem. Let $G°$ with appropriate subscripts represent the free energy of these various forms under standard conditions. At 25°, $\Delta G_1°$ and $\Delta G_2°$ are both positive, so that $G_b° > G_a°$ and $G_d° > G_c°$; moreover $\Delta G_1° > \Delta G_2°$ (Table 2) and $(G_d° - G_b°)$ is probably positive (see Appendix). Thus the order of decreasing free energies at 25° in the standard state (unit activity in solution) appears to be $d > b > a, c$. At higher temperatures, *e.g.*, 100°, there is evidence [19] that $G_b° > G_d°$, so that (*d*) and (*b*) would have to be interchanged.

The relative free energies of the ionised helical and the denatured form, (*a*) and (*c*), show some interesting features. The $G°$ terms represent the free energies in the standard state per mole of ionisable groups, *i.e.*, per mole of hydrogen-bonded base pairs (one

[19] Rice and Doty, *J. Amer. Chem. Soc.*, 1957, **79**, 3937.

proton-accepting group being assumed per hydrogen-bonding system). The earlier analysis has suggested that when two adjacent groups in the double helix are ionised and cease to be hydrogen-bonded then the helical configuration is permanently lost and the polynucleotide chain takes up its random configuration (*c*) in that region. This means that instead of the free energy of two adjacent ionised groups in the helical structure being $2G_a^\circ$, as for two isolated ionisations, it now becomes $G_c^\circ = (2G_a^\circ + \Delta G^\circ_{\text{config.}})$, where $\Delta G^\circ_{\text{config.}}$ is the change in free energy consequent upon the extra configurational freedom of the random chain in state (*c*). Since $\Delta H_{\text{config.}}$ will be negligible, no new bonds being formed or broken in the change (*a*) to (*c*), and $\Delta S_{\text{config.}}$ will be positive owing to the loss of order, $\Delta G_{\text{config.}}$ $(= \Delta H_{\text{config.}} - T.\Delta S_{\text{config.}})$ will be a negative quantity, and $G_a^\circ - G_c^\circ = -\Delta G_{\text{config.}}/2$. Thus the free-energy sequence can be tentatively deduced as $d > b > a > c$ at 25° and at other temperatures (below the critical denaturation point) where the β–γ relation is determined by $n = 2$ in equation (3). At these temperatures, the critical point in ionisation ($\gamma = \gamma_c$) presumably corresponds to a point where the *average* energy per base pair has been so enhanced by the extra rotational (etc.) energy of movement in the undenatured regions that it reaches, as it were, the critical " temperature " and the remaining hydrogen bonds between un-ionised groups break down. (This interpretation assumes that denatured and helical regions co-exist in the same molecule; but see below.)

With increase in temperature, the " equilibrium " between (*b*) and (*d*) may be regarded as shifting in favour of (*d*), according to the evidence of Rice and Doty [19] (*i.e.*, ΔH positive), and eventually $G_d^\circ < G_b^\circ$. The denaturation then appears to be thermal and not to involve ionised forms, although it has been suggested [20] that these are still necessary intermediates. Even if this is so, the thermal denaturation process is much more akin to the situation in the forward titration when $\gamma \geqslant \gamma_c$ than when $\gamma < \gamma_c$ where the $n = 2$ mechanism applies. Thus this latter mechanism need not be opposed to that thought [1,11,16] to prevail in purely thermal denaturation when longer sequences (10—100) of hydrogen bonds are regarded as being broken simultaneously. However, much more quantitative information is necessary on thermal denaturation before any detailed comparison can be properly made.

Most observers agree that the acid-denaturation process occurs extremely quickly and apparently coincidentally with ionisation.* The " free energy " of activation involved in the process (*a*) ⟶ (*c*) must therefore be very small and beyond the range of present methods of study. Again, this seems to be different from thermal denaturation for which small but finite " free energies " of activation, coupled with large heats and entropies of activation, have been provisionally deduced.[1,19,21]

In the foregoing, it has been tacitly assumed that the original double-helical molecule can be partly denatured so that double-helical (" crystalline ") regions and denatured disordered (" amorphous ") regions are juxtaposed within any one molecule (*e.g.*, ref. 17, Fig. 6, but with $n = 2$). It has however been suggested [22] that in the partially denatured state ($0 < \beta < 1$) some of the molecules are completely denatured and the rest remain intact. This may well be so after denaturation by heat, when the rupture of a fairly long sequence of hydrogen bonds seems to be the essential step and could then reasonably be expected to result in a complete breakdown of the rest of the molecule. However, the process of denaturation by ionisation differs from this in certain respects, notably in the character of the difference curves.[11] In particular, the agreement of the deduced β–γ

* The rapidity of acid-denaturation to a β value which then remains stable suggests that in the intact regions the continual interchange of protons scarcely ever produces new disordered sequences or, if so, only extremely slowly. This stability is not inconsistent with the titration curves of the helical structure, when allowance is made for the guanine ionisation, and may also be enhanced by unfavoured sequences of the bases in the remaining intact regions (see discussion in ref. 11, p. 776).

[20] Cavalieri, Rosoff, and Rosenberg, *J. Amer. Chem. Soc.*, 1956, **78**, 5239.
[21] Dekker and Schachman, *Pro. Nat. Acad. Sci. U.S.A.*, 1954, **40**, 894.
[22] Doty, comments on a paper by Peacocke and Preston, *J. Polymer Sci.*, 1958, **31**, 1.

plots with equation (3) for $n = 2$ is more consistent with partial denaturation within each molecule, on which equation (3) was based,[11] than with the alternative " all-or-nothing " possibility.

Appendix

The quantity $(\Delta G_2^\circ - \Delta G_1^\circ)$ has been discussed * in the preceding paper of this series,[6] where it was suggested that it can be regarded as the sum of a non-electrostatic term, independent of ionic strength (μ), and an " electrostatic " term which varies with μ. If subscripts (*a*)—(*d*) denote the same molecular species as in equations (1), (2), (4), and (5), and subscripts ν and μ the terms independent of and dependent upon μ, then

$$(\Delta G_2^\circ - \Delta G_1^\circ) = (G_d^\circ - G_c^\circ) - (G_b^\circ - G_a^\circ) =$$
$$[(G_a^\circ - G_c^\circ)_\nu + (G_d^\circ - G_b^\circ)_\nu] + [(G_a^\circ - G_c^\circ)_\mu + (G_d^\circ - G_b^\circ)_\mu] \quad . \quad . \quad (6)$$

where the G° are free energies under standard conditions, each equal to $[(G^\circ)_\nu + (G^\circ)_\mu]$.

At high μ the effects of interionic forces are minimal, so that a decrease of μ from 0·50 to 0·02 should increase the influence of the " electrostatic " factors and was observed to diminish $(\Delta G_2^\circ - \Delta G_1^\circ)$ by 1·8 kcal./mole, which means that it reduces the acidity of the helical form relative to that of the denatured state. The parallel displacement of the back-titration curves [5] with changing μ has already indicated [23] that the electrostatic potential at the amino-groups is determined by its immediate environment and depends very little on the configuration of the polynucleotide chain configuration, so that $(G_a^\circ - G_c^\circ)_\mu$ should be negligible. Hence the decrease in $(\Delta G_2^\circ - \Delta G_1^\circ)$ with decreasing μ must be attributed mainly to a decrease in $(G_d^\circ - G_b^\circ)_\mu$, which means that the free energy of (*b*) has increased relatively to that of (*d*), *i.e.*, the double-helical form has become relatively less stable at the lower ionic strengths. The same conclusion has also been reached on other grounds [5, 16, 21] and has been attributed to a weakening of the structure when the repulsion between the phosphate charges is enhanced at low μ.

The quantity $(\Delta G_5^\circ - \Delta G_4^\circ)$ can also be regarded as the sum of two factors, one independent of and the other dependent on μ, so that, with the same notation as in (6):

$$(\Delta G_5^\circ - \Delta G_4^\circ) = [(G_f^\circ - G_e^\circ)_\nu - (G_d^\circ - G_b^\circ)]_\nu + [(G_f^\circ - G_e^\circ)_\mu - (G_d^\circ - G_b^\circ)_\mu] \quad . \quad (7)$$

$(\Delta G_5^\circ - \Delta G_4^\circ)$ is negative at all μ, which means that the $^-NH{\cdot}CO^-$ groups in the denatured molecule [equation (6)] act as stronger acids than do the same groups when in the double helix. For high μ, when the " electrostatic " terms are negligible, this implies that, in the alkaline branches of the titration curves ($h < 0$), the complementary hydrogen bonds are stabilising, relatively to the denatured form (*d*), the helical form (*b*) which is acting as an acid [equation (5)]; in the acid branches ($h > 0$), these complementary hydrogen bonds again stabilise, relatively to (*d*), the helical form (*b*) which here, in contrast, acts as the conjugate base [equation (1)]. This is equivalent to saying that $(G_d^\circ - G_b^\circ)_\nu$ is positive; if this is so, $(G_a^\circ - G_c^\circ)_\nu$ and $(G_e^\circ - G_f^\circ)_\nu$ could be positive (which implies that the charged helical form has a higher free energy than the charged denatured form), or these quantities could be negative but numerically small in comparison with $(G_d^\circ - G_b^\circ)_\nu$. Rice and Doty [19] have calculated from the viscosity changes when sodium deoxyribonucleate is heated (at $\mu \sim 0{\cdot}15$) that, if it is assumed that (*b*) $\rightleftharpoons$ (*d*) at different temperatures, $\Delta H = H_d - H_b = 110$ kcal./mole and $\Delta S = S_d - S_b = 300$ cal./degree, which makes $\Delta G = G_d^\circ - G_b^\circ = +2{\cdot}1$ kcal./mole at 25°. Such calculations, based as they are only on viscosity measurements, are very provisional numerically, but they are consistent with the above suggestion that $(G_d^\circ - G_b^\circ)$ is positive at 25°.

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* *Erratum.* The values given in Part V [6] for this free-energy difference ($\Delta G_{III}^\circ - \Delta G_{IV}^\circ$ in the notation there) are incorrect (+200 to +480 cal./mole) and should read +1·0 to +2·5 kcal./mole.

[23] Peacocke and Lifson, *Biochim. Biophys. Acta*, 1956, **22**, 191.