521. *Electrometric Titration of the Sodium Salts of Deoxyribonucleic Acids. Part IV.* Denaturation by Heat in Aqueous Solution.*

By R. A. Cox and A. R. Peacocke.

A method is described whereby the fraction of hydrogen bonds in sodium deoxyribonucleate which have been permanently ruptured in any denaturing process may be determined from the progressive displacement of the forward titration curves over the pH range 7—3. This method, applied to the denaturation caused by heating solutions of herring-sperm deoxyribonucleate for one hour at various temperatures, indicated random rupture of the hydrogen bonds. No hydrogen bonds were broken until the temperature exceeded 75°, whereafter denaturation was extremely rapid. There was a linear relation between the fraction of hydrogen bonds permanently broken and the increase in ultraviolet absorption caused by heat.

When sodium deoxyribonucleate is heated in neutral aqueous solution, irreversible changes occur above a critical temperature which varies with the source of the deoxyribonucleate [1] and its method of extraction.[2a] These irreversible changes, which often take place over a temperature range of only a few degrees, include: a drop in the viscosity;[2-7] an increase in the ultraviolet absorption;[1,3,7] a displacement of the spectrophotometric titration curves;[8] the appearance of new infrared absorption bands;[9] changes in sedimentation constant;[2,5,6] and displacement of the titration curves.[10] Some investigators deduced a decrease in molecular weight from sedimentation and viscosity measurements after the nucleate had been heated in water,[5,11] and in salt,[6] whereas others reported [2,11] no change in molecular weight after heating in the presence of sufficient sodium chloride. A similar "protective" action of salt has also been observed in studies of the changes in ultraviolet absorption.[1]

In terms of the double-helical structure [12] for sodium deoxyribonucleate these physicochemical changes have been interpreted by several authors [1,2,5,7] as the consequence of

* Part III, *J.*, 2499.

[1] Thomas, *Biochim. Biophys. Acta*, 1954, **14**, 231.
[2] (*a*) Doty, Proc. 3rd Congr. Biochem., Brussels, Academic Press, New York, 1955, p. 135; (*b*) Doty and Rice, *Biochim. Biophys. Acta*, 1955, **16**, 446.
[3] Goldstein and Stern, *J. Polymer Sci.*, 1950, **5**, 687.
[4] Kurnick, *J. Amer. Chem. Soc.*, 1954, **76**, 417.
[5] Dekker and Schachman, *Proc. Nat. Acad. Sci. U.S.A.*, 1954, **40**, 894.
[6] Shooter, Pain, and Butler, *Biochim. Biophys. Acta*, 1956, in the press; ref. 2(*a*), p. 139.
[7] Laland, Lee, Overend, and Peacocke, *ibid.*, 1954, **14**, 356.
[8] Shack and Thompsett, *J. Biol. Chem.*, 1952, **197**, 17.
[9] Blout and Lenormant, *Biochim. Biophys. Acta*, 1955, **17**, 325.
[10] Cosgrove and Jordan, quoted by Jordan, *Progr. Biophysics Biophys. Chem.*, 1951, **2**, 81.
[11] Sadron, ref. 2(*a*), pp. 125, 134.

permanent rupture of the hydrogen bonds which hold the two helices together. When this process occurs without cleavage of covalent internucleotide linkages it has been described [1,2] as "denaturation," by analogy with the behaviour of proteins. However, the conclusion that hydrogen bonds are ruptured by heat is not unambiguous since the properties which have usually been measured depend only indirectly on the presence of hydrogen bonds. Since the anomaly in the deoxyribonucleate titration curves is directly caused by the hydrogen bonds between titratable groups and since the accuracy and reproducibility of the continuous titration curves have recently been established,[13] this method should afford a suitable criterion of denaturation. A brief report [10] has already indicated that ionisable groups can be released by heating deoxyribonucleate solutions at 80° for 5—10 minutes.

In the following, a method of determining from titration curves the proportion of hydrogen bonds ruptured by any process is described and is used to measure the effects of heating herring-sperm sodium deoxyribonucleate in solution for one hour at various temperatures. A quantitative method for measuring such denaturation seems desirable in view of the close association between the intact hydrogen-bonded structure and its biological activity as demonstrated [14] by the simultaneous decrease in both transforming activity and viscosity when solutions of the deoxyribonucleate transforming principle from *H. influenza* are heated at temperatures above 81°.

Experimental and results

The isolation and properties of the herring sperm sodium deoxyribonucleate have been described.[7,13] (Light scattering measurements made in this laboratory by Mr. B. N. Preston now show that this sample has a molecular weight of $6{\cdot}3 \times 10^6$.) Solutions of the nucleate (0·142 mg. of phosphorus/ml.; approx. 0·15%) in 0·05M-sodium chloride, pH 6—7, were heated for 1 hr. at atmospheric pressure in glass-stoppered 100 ml. Pyrex flasks in a water-bath at 45°, 70°, 75°, 79°, 83·4°, 87·5°, and 100° ± 0·2°. There was no significant change in the nucleate concentration during heating. The solutions, still at pH 6—7, were then titrated at 25° to pH 2·75 and then back-titrated to pH 7·5—8. The method employed was that of continuous titration in a cell with liquid junction at an ionic strength of 0·05 (the small contribution of the nucleate being neglected). pH was measured with a glass electrode and the general procedures were those described previously, when their reliability and limitations were also established.[13]

In Fig. 1 the continuous line of curve I represents the forward titration curve obtained at 25° on addition of acid to the original unheated nucleate. Along this curve the amino-groups of, first, cytosine and then adenine are successively ionised with disappearance of their associated hydrogen bonds. This removal of hydrogen bonds appears to be permanent and irreversible when they occur in sequences sufficiently long for their loss to destroy the original helical configuration. The process of acid denaturation is complete by pH 3·0 and thereafter any further addition of acid or alkali affords the titration curve (II) of the completely denatured non-hydrogen-bonded polynucleotide chain. Line II of Fig. 1 is this back-titration curve for the unheated herring-sperm sodium deoxyribonucleate. Subtracting the alkali bound along II from that bound along I gives the difference curve (I − II).

Titration at 25° of the nucleate after it had been heated for 1 hr. at 45°, 70°, and 75° yielded forward- and back-titration curves similar to I and II, except that there was a slight displacement along the ordinate scale which was constant over the whole pH range. This displacement was small (<0·08 unit on the ordinate scale of Fig. 1) and differed in duplicate experiments at the same temperature. It was therefore attributed to reaction of the hot solvent with the glass vessel (*e.g.*, replacement of a few hydrogen ions by sodium ions). Consequently, in order to allow comparison of the various titration curves, they have been displaced slightly along the ordinate scale so that the inflexion points of the back-titration curves fall on the zero of the ordinate scale. This correction allows fully for any slight uptake of alkali from the glass and, for any given sample, is the same for all pH's, so that it does not affect the shape of the titration or difference curves. The titration curves of the nucleate heated for an hour at 45°, 70°, and

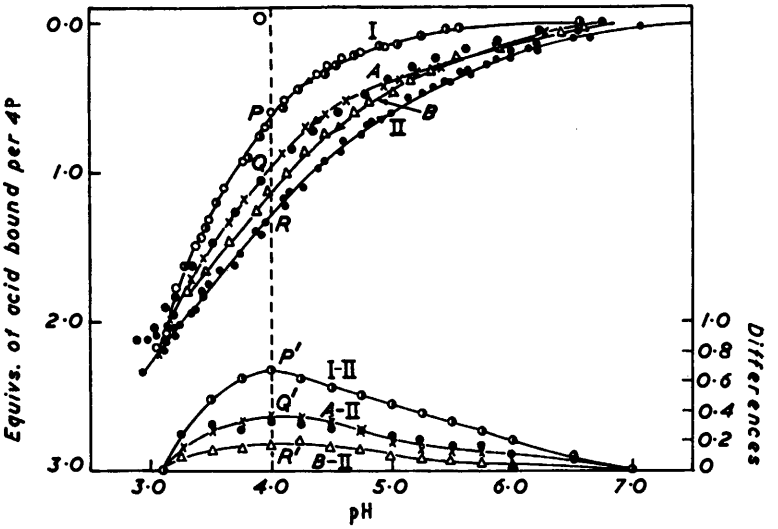
[12] Watson and Crick, *Nature*, 1953, **171**, 737, 964; Wilkins, Stokes, and Wilson, *ibid.*, p. 738; Franklin and Gosling, *ibid.*, 1953, **172**, 156.
[13] Cox and Peacocke, *J.*, 1956, 2499.
[14] Zamenhof, Alexander, and Leidy, *J. Exp. Med.*, 1953, **98**, 373.

75° then coincided with those of the original unheated sample and had the same difference curve (I—II).

Forward-titration with acid of nucleate solutions heated for 1 hr. at higher temperatures (79°, 83·4°, 87·5°) yielded curves (*A*, *B*) intermediate between I and II. Nevertheless on back-titration from pH 2·75, the same curve (II) was obtained as with the original unheated solutions. The nucleate solution which had been heated for 1 hr. at 100° showed no titration anomaly, forward-titration with acid and back-titration with alkali both yielding the single curve II. It follows that heating for 1 hr. can partly, at 79—87·5°, and wholly at 100°, have the same effect on the nucleate structure as ionisation of the amino-groups during titration with acid. So heating must progressively destroy the hydrogen bonds in the original double-helical structure. The displacement of the titration curves which accompanies this process is seen in the sequence I, *A*, *B*, and II and is also illustrated by the change in the difference curves (Fig. 1, lower part). The quantitative interpretation of these displacements is discussed below.

FIG. 1. *The effect of previous heat treatment on the titration curves of herring-sperm sodium deoxyribonucleate.*

Titration conditions: Solvent, 0·05M-NaCl; 25°; nucleate concn., 0·141 mg. P/ml.



Upper figure. Left ordinate scale.

Solid lines I and II: forward- and back-titration curves, respectively, of the original, unheated nucleate.
Points on the forward-titration curves of nucleate solutions (0·15% in 0·05M-NaCl) previously heated for 1 hr. at various temperatures: ○ 70°; ◑ 75°; × 78·8°; ● 83·4°; △ 87·5°.
Smaller full circles (•): points on the back-titration curves of all the heated solutions and also on the forward-titration curve of the solution previously heated for 1 hr. at 100°.

Lower figure. Difference curves. Ordinate scale, lower right. Points marked obtained by interpolation in the upper figure.

△ = alkali bound along the forward-titration curves minus alkali bound along curve II (in equivs./4P atoms). *O*, *P*, *Q*, *R*, *P'*, *Q'*, *R'*, see text.

No increase was observed in the number of titratable groups over the pH range 5—9. Hence there was no detectable release of secondary phosphoryl end groups because of the heat treatment—although it must be realised that the titration method is very insensitive to rupture of internucleotide linkages, the production of one such new group for every 50 phosphorus atoms being scarcely detectable.

After being heated, a portion of each nucleate solution was diluted with 0·05M-sodium chloride and its ultraviolet extinction was measured at 260 mμ at pH 7·5—8 relatively to a 0·05M-salt solution. An increase in absorption occurs when the temperature of heating is 79° or higher and is a maximum at 100°. The increments in the extinction are presented in Table 1 as percentages of this maximal increase.

Determination of the Nature and Extent of Denaturation from the Displacement of the Titration

Curves.—Quantitative interpretation of the displacement, with heating, of the forward-titration curves (Fig. 1) will next be considered.

Let β represent the fraction of the hydrogen bonds originally present which have been ruptured in a random manner before titration; that is, we assume that the hydrogen bonds linking the pair of bases adenine and thymine have been ruptured to the same extent as those linking guanine and cytosine. The resulting deoxyribonucleate molecule may then be regarded as composed of regions where all the hydrogen bonds are intact and of denatured, disordered

TABLE 1. *Effects of heating herring-sperm sodium deoxyribonucleate on its ultraviolet absorption and hydrogen bonding.*

Temp. of heating for 1 hr. in 0·05M-NaCl	$100\Delta\varepsilon/\Delta\varepsilon_{max.}$*	Hydrogen bonds ruptured (%) (100β) †
70°	0	0
75	5	0
78·8	55	47
83·5	50	47
87·5	74	73
100	100	100

* $\Delta\varepsilon$ = Increase in extinction at 260 mμ due to heating; $\Delta\varepsilon_{max.}$ = $\Delta\varepsilon$ after heating at 100°.
† β deduced from titration curves by using eqn. (1), see Table 2.

regions containing no hydrogen bonds, the number of nucleotides in these two regions being in the ratio $(1 - \beta) : \beta$. If, as seems likely, these regions dissociate independently, the resulting forward-titration curve with acid should be the same as that given by a mixture of two distinct molecular species, namely, the intact fully hydrogen-bonded molecules of titration curve I and the completely denatured molecule of titration curve II. Such a mixture would have to contain the same total concentration of nucleotides divided in the ratio $(1 - \beta) : \beta$ between the two types of molecule. At any given pH on Fig. 1, let P, R be points on curves I and II and let Q be the corresponding point on the forward-titration curve of the mixture just defined (in Fig. 1, Q is shown as a point on the experimental curve A). Then OP, OR, and OQ represent, respectively, the equivalents of acid bound per 4 g.-atoms of phosphorus by the intact molecule, the completely denatured molecule, and the mixture, *i.e.*, the molecule with β of its hydrogen bonds ruptured. The composition of the mixture implies that

$$OQ = \beta OR + (1 - \beta)OP$$

whence it follows that

$$\beta = PQ/PR \text{ and } (1 - \beta) = QR/PR \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

This has been deduced for one particular pH, but in *random* rupture of hydrogen bonds, although the groups involved titrate in different ranges, the value of β would not vary with pH.* So the titration curve of the mixture, and hence of the nucleate with a fraction (β) of its hydrogen bonds ruptured, is the locus of the point Q, defined by $PQ/PR = \beta$, a constant at all pH's. A plot of the difference between this curve and curve II will then have the same shape as (I—II) of Fig. 1, but with the ordinates all reduced by the factor β; P', Q', and R' on these difference curves in the lower part of Fig. 1 correspond to P, Q, and R on the titration curves. These conclusions about the disposition of the curves follow in spite of the irreversibility of the forward-titration curve of any sample still containing hydrogen bonds. The course of such a titration is governed only by the fraction (β) of hydrogen bonds randomly ruptured before the titration begins; the titration then completes the denaturation. Thus the back-titration curves all represent the dissociation of the completely non-hydrogen-bonded nucleate and so cannot be used to find the value of β due to heating.

The rupture of hydrogen bonds in the process occurring before titration may be non-random, those linking the base pair adenine–thymine, for example, breaking more slowly than those linking guanine–cytosine. Then, in any given range pH to [pH + d(pH)], PQ/PR would still be equal to the ratio of acid bound by the groups titrating * in this range in the denatured and the intact regions of the molecule but it would vary over the titration curve. The fraction of *all* the

* In both the random and the non-random case, the groups titrating at a given pH are not the *same* groups in the intact and the denatured regions. This does not affect the deduction of eqns. (1), (2), and (3) which describe the distribution of hydrogen ions between the two regions, without any need for specification of the chemical nature of the groups to which the ions are attached.

hydrogen bonds originally present which had been permanently broken by the treatment before titration could then be defined without regard to the nature of the groups in question and denoted by $\bar{\beta}$. This quantity could only be obtained by a summation and averaging of the ratio PQ/PR over all the potentially hydrogen-bonded groups titrated so that

$$\bar{\beta} = \left(\sum_n PQ/PR\right)/n \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

where n is a convenient finite number of points on the experimental forward-titration curve, which have been chosen so as to subdivide the ordinate scale into intervals of equal amounts of acid or alkali bound by the adenine and cytosine amino-groups only. The summation in eqn. (2) is then taken over all these n points.* The subdivisions so defined are also equal subdivisions of the ordinate scale over most of the experimental titration curves, since only at the lower end do any groups titrate which cannot be hydrogen-bonded, *viz.*, the guanine 2-amino-groups.

The greater is the number n the more accurate is the value of $\bar{\beta}$ and in the limit

$$\bar{\beta} = \int (PQ/PR) \,.\, \mathrm{d}h \Big/ \int \mathrm{d}h \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

where h represents the alkali or acid bound by adenine and cytosine groups and the integration is again taken over the h range of titration of these groups (*i.e.*, over h = 0—2·0 equivs. of acid bound per 4 P).

Discussion

The above treatment has been applied to the curves A and B of Fig. 1, and Table 2 shows the values for the ratio PQ/PR at various points along the curves (chosen in this

Table 2. *Deduction of fraction of hydrogen bonds ruptured from titration curves.*

	PQ/PR *			PQ/PR *	
pH	Curve *A*, Fig. 1 (78·8° & 83·5°)	Curve *B*, Fig. 1 (87·5°)	pH	Curve *A*, Fig. 1 (78·8° & 83·5°)	Curve *B*, Fig. 1 (87·5°)
3·25	0·42	0·67	4·25	0·46	0·72
3·5	0·46	0·77	4·5	0·45	0·71
3·75	0·47	0·74	4·75	0·48	0·72
4·0	0·47	0·73	5·0	0·53	0·75
			β = mean value of PQ/PR =	0·47	0·73

* See text and Fig. 1 for definition.

case at equal intervals of pH). In the range of the curves where the accuracy is greatest PQ/PR is reasonably constant for both A and B and therefore indicates random breakage of the hydrogen bonds during the heating. The mean values of this ratio give the fraction, β, of hydrogen bonds ruptured at 78·8° and 83·5° as 0·47 and at 87·5° as 0·73, where both figures have standard deviations of 0·03. These values for β are approximately equal to the increases in ultraviolet absorption when these are expressed as a fraction of the maximum increase. There is therefore a linear relation between these quantities when hydrogen bonds are broken randomly. This now confirms earlier suggestions [1,7] that these changes in ultraviolet absorption are a direct consequence of breakdown of the hydrogen bonds holding together the two helices.

The fraction of hydrogen bonds permanently ruptured after 1 hour's heating is plotted in Fig. 2 as a function of the temperature of heating. Thomas [1] has tentatively shown that on heating at different temperatures the overall ultraviolet absorption increase is the same, which suggests that, once begun, denaturation eventually goes to completion. If further experimental studies confirm this, the β values plotted in Fig. 2 must be regarded as interpolations at one hour in a series of curves of β against time of heating. The kinetics of the heat denaturation process awaits further study but if the process is assumed

* This method of obtaining β by eqn. (2) was employed, without explanation, in an earlier publication [15] on the effect of γ-rays on deoxyribonucleate solutions.

[15] Cox, Overend, Peacocke, and Wilson, *Nature*, 1955, **176**, 919.

to be of the first order (as with many proteins [16]) with respect to the concentration of native hydrogen-bonded nucleate, then $\ln [1/(1 - \beta)] = kt$ and the first-order rate constant, k, can be calculated. The present studies are inadequate for accurate determinations of k, but on the above assumptions k increases from $1{\cdot}0 \times 10^{-2}$ to $2{\cdot}2 \times 10^{-2}$ min.$^{-1}$ over the temperature interval 83·5—87·5°. This implies an activation energy of the order of 50 kcal. for the denaturation of the helical structure and may be compared with the approximate value of 60 kcal. obtained [5] from earlier ultraviolet absorption [1] and viscosity [4] results, and the values reported by Doty and Rice [2] of 36—93 kcal. These high activation energies seem to be analogous to those obtained for the denaturation of proteins [16] and strongly suggest the need for the simultaneous rupture of sequences of hydrogen bonds. Up to 75°, heating for 1 hour in solution apparently does not produce sequences of broken

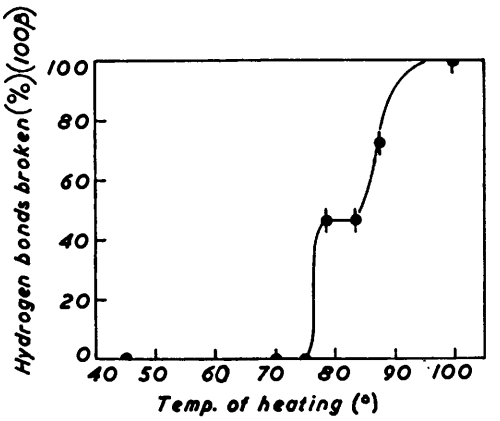


FIG. 2. *The rupture of hydrogen bonds in herring-sperm sodium deoxyribonucleate by heating of an aqueous solution* (0·15%; 0·05M-NaCl) *for* 1 *hr.* β *calculated from titration curves of Fig.* 1 (*see Tables and text*).

hydrogen bonds sufficiently long for the bonds not to be re-formed. But above this temperature, the sequences are long enough for the double-helical structure to be permanently distorted and so never to regain its original ordered condition.

The near-equality of the fraction of hydrogen bonds broken at 78·8° and 83·5° suggests a small step in the curves of Fig. 2 as though the denaturation proceeds in two distinct stages. A similar observation has been reported [1] for the change, on heating, of the ultraviolet absorption of several deoxyribonucleate preparations from different sources. In view of the titration evidence for the random nature of the heat-denaturation process, this apparent step cannot readily be interpreted in terms of selective rupture of hydrogen bonds between particular groups. The observations may arise from the undoubted heterogeneity [17] of all deoxyribonucleate preparations, different components having perhaps different stabilities towards heat.

The present studies clearly show that one of the main effects of heating saline solutions of herring-sperm sodium deoxyribonucleate is to cause the permanent breakdown of the hydrogen-bonded structure and that by means of titration the extent of this denaturation can be measured.

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CHEMISTRY DEPARTMENT, THE UNIVERSITY,
EDGBASTON, BIRMINGHAM, 15. [*Received, February 7th,* 1956.]

[16] Glasstone, Laidler, and Eyring, " The Theory of Rate Processes," McGraw-Hill Book Co., New York, 1941, p. 442 *et seq.*; Putnam, " The Proteins," ed. Neurath and Bailey, Academic Press, New York, Vol. I, Part 3, pp. 860 *et seq.*

[17] Chargaff, Crampton, and Lipshitz, *Nature*, 1953, **172**, 289; Brown and Watson, *ibid.*, p. 339.