953. *Electrometric Titration of the Sodium Salts of Deoxyribonucleic Acids. Part V.* The Effect of Temperature.*

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

The dissociation curves in 0·05M-sodium chloride of herring-sperm sodium deoxyribonucleate have been studied at different temperatures by use of a continuous titration method. The reversible dissociation curves of sodium nucleate completely denatured by prior acid treatment varied with temperature and approximate values of the apparent heats of dissociation for the different titratable groups were deduced. The acid denaturation of sodium nucleate, as shown by the relative dispositions of the forward- and back-titration curves, depends on temperature and evidence is presented that sodium nucleate is only partly denatured by titration with acid to pH 2·6 at 0·4°. The reversible dissociation curve at 0·4° of the undenatured double-helical structure has been computed.

The titration curves of sodium deoxyribonucleate are anomalous, the forward-titration curve being irreversible and different from the reversible back-titration curve. The initial forward-titration with acid or alkali from pH 7 causes a permanent and irreversible rupture of hydrogen bonds, or " denaturation," through ionisation of the groups involved in them, whereas the subsequent back-titration from pH 2·5 or 12 causes reversible changes only in the state of ionisation of the molecule.[1] Hitherto the titrations have been carried out at 25° but curves obtained at other temperatures should be helpful in two respects: first, the displacement of the reversible back-titration curves with temperature should yield [2] the apparent heats of dissociation as a function of pH and thereby indicate the pH ranges in which the various groups dissociate; and secondly, the effect of temperature on the irreversible forward-titration curves might help to elucidate the mechanism of denaturation of the nucleate by acid. The titration behaviour of herring-sperm sodium deoxyribonucleate at 0·4°, 25°, and 35° is described and analysed here chiefly with the first aim. Its significance with respect to denaturation will be examined subsequently (a preliminary account has been given [3]).

Experimental

The sample of herring-sperm sodium deoxyribonucleate was that described in preceding Parts.[1,4] Solutions of the nucleate (0·141 mg. of phosphorus/ml., about 0·2%) in sodium chloride (0·05M) were titrated at the appropriate temperature with acid to a pH just below 3 and then back-titrated with alkali. The results at 25° have been reported.[1] The method of continuous titration in a cell with liquid junction and glass electrode was employed with the procedure and precautions already described.[1] The temperature was controlled by a water-bath maintained at 0·4° ± 0·2°, 25° ± 0·05°, or 35° ± 0·05°. Solutions of hydrochloric acid (0·1M), potassium hydrogen phthalate (0·05M), and sodium borate decahydrate (0·05M) were used to calibrate the glass electrode system and were assigned respectively the pH values [5] of $1{\cdot}08_5$, 4·01, and 9·39 at 0·4° and $1{\cdot}08_2$, $4{\cdot}02_5$, and 9·10 at 35°.

Results.—The titration behaviour at 0·4° is shown in Fig. 1, where curve I is the irreversible forward-titration curve and II the corresponding reversible back-titration curve from the end-point of I. Compared with the reversible back-titration curve at 25°, curve II was displaced to much lower pH values than expected from the low values of the heats of dissociation subsequently deduced (see p. 4727). This, and the relatively small difference between curves I

* Part IV, *J.*, 1956, 2646.

[1] Cox and Peacocke, *J.*, 1956, 2499.
[2] Wyman, *J. Biol. Chem.*, 1939, **127**, 1.
[3] Cox and Peacocke, *J. Polymer Sci.*, 1957, **23**, 765.
[4] Cox and Peacocke, *J.*, 1956, 2646.
[5] Steinhardt and Harris, *J. Res. Nat. Bur. Stand.*, 1940, **24**, 335; 1940, **25**, 519; Harned and Owen, " The Physical Chemistry of Electrolytic Solutions," Reinhold Publ. Corp., New York, 1943, p. 321; Hitchcock and Taylor, *J. Amer. Chem. Soc.*, 1938, **60**, 2710.

and II, suggested that II was not the reversible dissociation curve at 0·4° of completely denatured nucleate but that some hydrogen bonds had re-formed when the charge on the amino-groups ($pK_a' < 7$) was removed during back-titration. Confirmation of this was obtained in two ways. (i) The nucleate solution at pH 6—7 obtained by a complete titration cycle at 0·4° to and from pH 2·6 was submitted to a further titration cycle to and from pH 2·7 at 25° (Fig. 2). In this second, 25° cycle the forward- and back-titration curves differed (V,

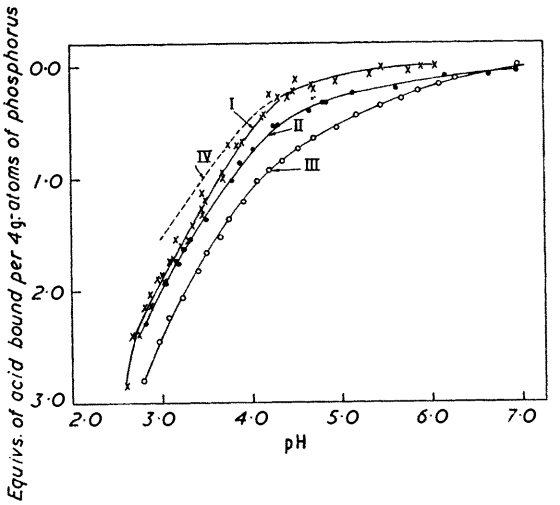
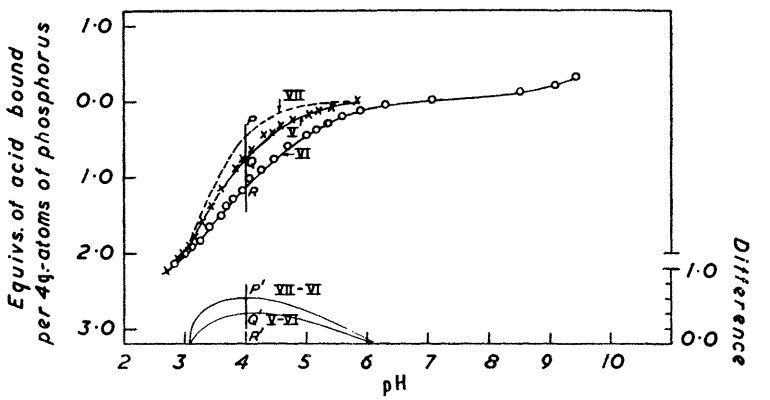


FIG. 1. *Titration curves of sodium deoxyribonucleate at* 0·4° *in* 0·05M-*sodium chloride.*

I, × Forward-titration with acid from pH 6·1. II, ● Back-titration with alkali from pH 2·6. III, ○ Titration with acid at 0·4° of a sodium nucleate sample which had previously been titrated at 25° from neutrality to pH 2·75 and back to pH 7. IV, – – – Computed reversible forward-titration curve of sodium nucleate in the double-helical configuration.

FIG. 2. *Titration curves at* 25° *in* 0·05M-*sodium chloride of sodium deoxyribonucleate previously titrated at* 0·4°.



V, × Forward-titration with acid to pH 2·75 of a nucleate sample which had been titrated at 0·4° from neutrality to pH 2·4 and back.

VI, ○ Back-titration from pH 2·75 on completion of V. VI is also the usual back-titration curve at 25° of completely denatured nucleate.

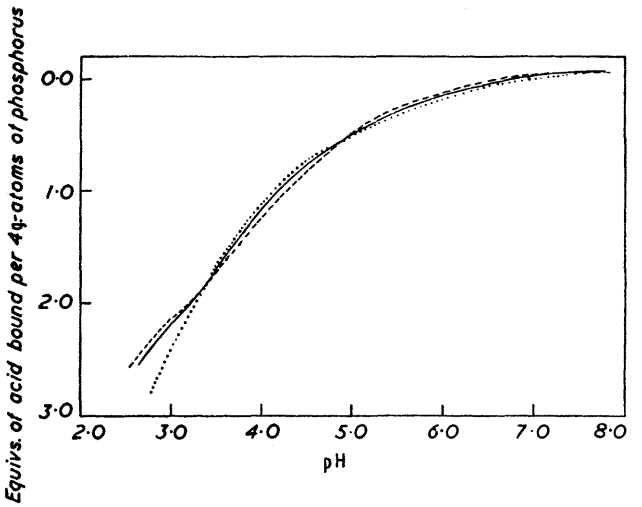
VII, – – – – Forward-titration curve with acid of nucleate not previously titrated at 0·4°.

Lower portion. Difference curves; Δ = Alkali bound along V or VII *minus* alkali bound along curve VI, ordinate scale on lower right. P', Q', and R' in the lower figure correspond to P, Q and R, respectively, on the titration curves.

VI, Fig. 2). From the position of V relative to VI and to VII, the forward-titration curve at 25° of a completely hydrogen-bonded nucleate, it was deduced by the method previously described [4] that this material still retained 60% of its original hydrogen bonds after having undergone the titration cycle at 0·4°. This is also illustrated by the lower comparison curves of Fig. 2 in which the ratio $Q'R'/P'R'$ was 0·6 at all pH values. (ii) The reversible dissociation curve at 0·4° of a completely denatured nucleate (III, Fig. 1) was obtained by first removing all

hydrogen bonds by a full titration cycle (to and from pH 2·75) at 25° and then repeating this at 0·4°, when forward- and back-titration both yielded the reversible curve III. The difference between II and III confirmed that II, which was also reversible, was the dissociation curve of a nucleate in which 60% of the original hydrogen bonds could re-form during back-titration from pH 2·6. Apparently at 0·4° there was an upper limit of 40% to the extent of permanent denaturation which ionisation of the amino-groups ($pK_a' < 7$) could cause.

FIG. 3. *Reversible titration curves of denatured sodium deoxyribonucleate in* 0·05M-*sodium chloride at various temperatures.*



. . . . Titration with acid at 0·4° of a solution of nucleate which has previously undergone a complete titration cycle at 25°.
——— Back-titration with alkali from pH 2·5, at 25°.
– – – – Back-titration with alkali from pH 2·6, at 35°. Each curve is the mean of several titrations.

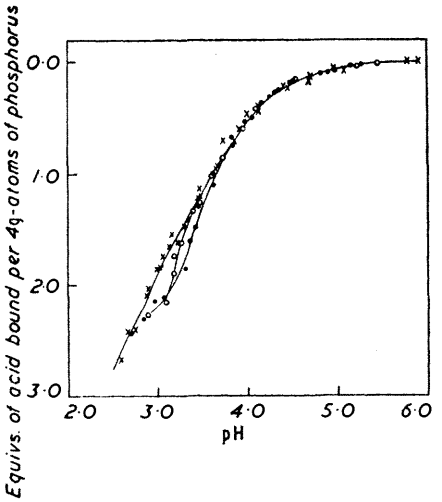


FIG. 4. *Forward-titration curves of sodium deoxyribonucleate in* 0·05M-*sodium chloride at various temperatures.*

× Forward-titration with acid from pH 6·0 at 0·4°.
○ Forward-titration with acid from pH 5·75 at 25·0°.
● Forward-titration with acid from pH 5·6 at 35·0°.

At 35° the curve obtained on back-titration from pH 2·6 was only slightly displaced from that at 25° (Fig. 3). The displacement was of the order expected from the apparent heats of dissociation deduced [6] for the similar dissociating groups in ribonucleic acids, and contrasted with that observed on lowering the temperature to 0·4°, as discussed above. At 35° the reversible back-titration curve clearly corresponded to a nucleate in the same denatured state

[6] Vandendriessche, *Compt. rend. Trav. Lab. Carlsberg*, 1951, **27**, No. 15, 341.

as that during the back-titration at 25°, since a rise in temperature of 10° should have made denaturation by ionisation easier: the displacement at 35° was therefore the usual effect of temperature on a reversible equilibrium. The forward titration curves at 0·4°, 25°, and 35° are compared in Fig. 4. In the first stages of ionisation from pH 3·75 to 6 there was little temperature effect, so that the heats of dissociation of the hydrogen-bonded groups in this first stage, and the temperature coefficient of denaturation, must both have been small (or exactly compensatory). But after 0·8 equivalent of acid per 4 g.-atoms of phosphorus had combined, the curves for the different temperatures diverged and for a given value of the protonic charge the number of groups released from hydrogen bonds increased with temperature. This was shown by the steeper approach at 35° than at 25° of the forward- to the back-titration curve. At 25° the forward- and back-titration curves met and denaturation was complete when 2·12 equivalents of acid per 4 g.-atoms of phosphorus had combined; but at 35° this occurred when only 1·86 equivalents had combined, and this was less than the total number (1·97 per

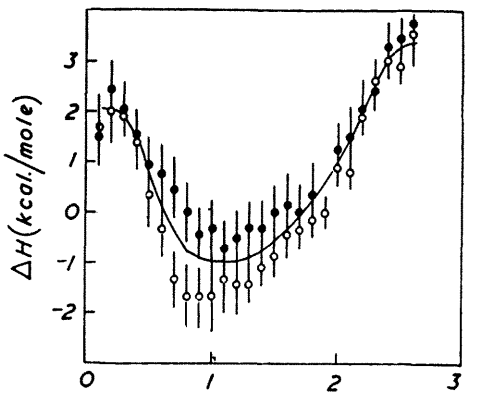


FIG. 5. *Dependence of the apparent heat of dissociation* ($\Delta H'$) *of sodium deoxyribonucleate on the acid bound* (h).

Computed from the reversible back-titration curves: ● for 0·4° and 25·0°, ○ for 0·4° and 35·0°.

Vertical lines represent uncertainty in $\Delta H'$.

4 g.-atoms of phosphorus) of hydrogen-bonded cytosine and adenine amino-groups originally present. Apparently at 35° complete ionisation of all the hydrogen-bonded groups was not necessary for complete denaturation by acid. This must have been an effect of temperature changes on denaturation by ionisation and not a direct effect on the intact hydrogen-bonded structure since, at pH 6—7, heating caused no denaturation [4] in 1 hr. unless the temperature exceeded 75°.

Heats of Dissociation.—The displacement with temperature of the curves (Fig. 3) representing the reversible dissociation of completely denatured nucleate yields [2] the apparent heats of dissociation, $\Delta H'$, of the various groups since

$$\Delta H' = -2{\cdot}303RT^2\,[\partial(\mathrm{pH})/\partial T]_h \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

where $[\partial(\mathrm{pH})/\partial T]_h$ represents the rate of change of pH with temperature at a constant value of h, the number of equivalents of acid bound. Values of $\Delta H'$ were calculated from the curves in Fig. 3 for the temperature changes 0·4—25° and 0·4—35° by using the approximation [2]

$$\Delta H' = -2{\cdot}303RT_1T_2\{(\mathrm{pH}_2 - \mathrm{pH}_1)/(T_2 - T_1)\}_h \quad . \quad . \quad . \quad . \quad . \quad (2)$$

where subscripts 1 and 2 refer to two different temperatures ($T_2 > T_1$). The $\Delta H'$ values for the non-hydrogen-bonded nucleate are shown in Fig. 5 as a function of h. The values of $\Delta H'$ deduced from Fig. 3 for the various pH ranges, and hence for the various chemical groups, are summarised in the Table.

DISCUSSION

It has been demonstrated that curve II (Fig. 1) obtained at 0·4°, after forward-titration at 0·4° to pH 2·6, represents the reversible dissociation curve of a nucleate in which 40% of the hydrogen bonds have been *permanently* ruptured but in which 60% can re-form during back-titration, as shown by its subsequent behaviour at 25° (Fig. 2). These observations can be interpreted in terms of the double-helical model as follows. Only

those regions of the nucleate molecule which, in spite of the ionisation of the amino-groups and disappearance of the hydrogen bonds, retain their double-helical configuration would be expected to be able to re-form complementary hydrogen bonds when the amino-groups are discharged during back-titration. In contrast, amino-groups in disordered regions which have lost the double-helical configuration would not be able to re-form the complementary hydrogen bonds on back-titration.

On this basis, curve II (Fig. 1) can be interpreted as the composite reversible dissociation curve at 0·4° of two types of region in the nucleate ions: intact double-helical regions containing 60% of the bases, and denatured, presumably disordered, regions containing 40% of the bases. This implies the existence of a *reversible* dissociation curve for a double-helical structure; along this curve the double-helical form would be approximately retained as the amino-groups ionised and complementary hydrogen bonds would re-form when the pH rose. At 25° and 35° little indication of the existence of this curve is obtained since denaturation follows quickly upon ionisation as soon as the pH is lowered. At any given pH let p, q represent the equivalents of acid bound (per 4 g.-atoms of phosphorus) along the *reversible* dissociation curves at 0·4° of the double-helical and of the completely denatured forms, respectively. Then the equivalents, r, of acid bound reversibly (per 4 g.-atoms of phosphorus) by a nucleate containing 60% of its nucleotides in double-helical regions and 40% in denatured ones is given by $r = 0{\cdot}6p + 0{\cdot}4q$. The quantities r, q are given as functions of pH by curves II and III (Fig. 1), respectively, so p can be deduced and is plotted as curve IV (Fig. 1). Presumably, if titrations could be made at sufficiently low temperatures, curve IV might be directly observed on titration with acid, no " anomaly " would be obtained, and no denaturation would be caused by ionisation. The helical structure would, as it were, be " frozen " into stability even when all the groups involved in hydrogen bonds were ionised.*

The reversible dissociations occurring along the curves III and IV can be represented as follows:

Curve III

$$\{\text{Denatured chain}\}\text{–}NH_3^+ + H_2O \rightleftharpoons \{\text{Denatured chain}\}\text{–}NH_2 \cdots (H_2O) + H^+ \quad . \quad (3)$$

Curve IV

$$\{\text{Helix}\}\text{–}NH_3^+ + X\text{–}\{\text{Helix}\} \rightleftharpoons \{\text{Helix}\}\text{–}NH_2 \cdots X\text{–}\{\text{Helix}\} + H^+ \quad . \quad (4)$$

$-NH_3^+$ and $-NH_2$ are the charged and uncharged forms of the amino-groups capable of being hydrogen-bonded (adenine and cytosine 6-amino-groups [7a] and, possibly,[7b] the guanine 2-amino-group), X is the system to which these groups are hydrogen-bonded in the double helix (the 1 : 6 –NH·CO– systems of thymine and guanine according to the X-ray [7a] and titration evidence,[1] and possibly the cytosine 2-oxo-group [7b]), H_2O is a solvent water molecule, the terms enclosed in braces represent the corresponding polynucleotide chains and the dotted lines are hydrogen bonds of the specific double-helical type [7] in eqn. (4) and of a non-specific type involving solvent in eqn. (3). Let the apparent acidic dissociation constants and apparent standard free energies of dissociation of the amino-groups which correspond to curves III and IV be denoted by K_{III}', K_{IV}' and by ΔG_{III}°, ΔG_{IV}°, where $\Delta G^\circ = -\boldsymbol{RT} \ln K' = 0{\cdot}4343\ \boldsymbol{RT}.pK'$, with the appropriate subscripts. Then $\Delta G_{III}^\circ - \Delta G_{IV}^\circ = 0{\cdot}4343\boldsymbol{RT}(pK_{III}' - pK_{IV}') = 0{\cdot}4343\boldsymbol{RT}(pH_{III} - pH_{IV})_h$, where the last term refers to the pH displacement between curves III and IV at constant h.†

* In recent experiments at −0·4° the reversible dissociation curve of the double-helix in 0·05M-NaCl has been obtained and proved to be identical with curve IV (A. R. Peacocke and B. N. Preston, unpublished work, 1957; A. R. Peacocke, Symp. on Phosphoric Esters, Chem. Soc. Anniv. Meeting, Cambridge, 1957, Special Publication, in the press).

† Since comparison is made at constant h and only the *differences* in pK_a' and ΔG° between curves III and IV are employed, these equations, and what follows, apply to all the amino-groups dissociating at the particular h value.

[7] (*a*) Watson and Crick, *Nature*, 1953, **171**, 737; Peacocke, *Ricerca sci.*, 1955, **25**, 812; (*b*) Pauling and Corey, *Arch. Biochem. Biophys.*, 1956, **65**, 164.

It is clear from Fig. 1 that $K_{IV}' > K_{III}'$ but the interpretation below pH 3·75 is complicated by the presence in both III and IV of the dissociation of the 2-amino-group of guanine which, contrary to earlier ideas,[7a] may be hydrogen-bonded in the helical form.[7b] At pH values above 3·75 ($\Delta G_{III}^\circ - \Delta G_{IV}^\circ$) has values of +200 to +480 cal. per mole of hydrogen ions. This difference in the free energy of dissociation in the double-helical and denatured configurations can be regarded as the sum of two terms:

(i) A term which represents the differences in the non-electrostatic parts of the free energies of dissociation of the hydrogen ions in the two configurations. The most important contributor to this term would be the difference of free energy between the hydrogen bonds on the 6-amino-groups when they are linked to the 1 : 6 $^-$NH·CO$^-$ systems in the double-helix and when they are linked to solvent molecules in the denatured form.

(ii) An electrostatic term representing the effects in 0·05M-sodium chloride of different electrostatic potentials at the dissociating groups in the two configurations. The magnitude of this term in 0·05M-sodium chloride can be estimated by comparing the displacements of the reversible back-titration curves (denatured form) and of the reversible portions, at low h, of the forward-titration curves (double-helical form) for a change in sodium chloride concentration from 0·05M to 0·50M, when electrostatic effects are almost completely suppressed. The two displacements differed by 0·2 pH unit which corresponds to a contribution to ($\Delta G_{III}^\circ - \Delta G_{IV}^\circ$) of the order of 50 cal. per mole (these figures were derived from 25° curves but should give a reasonable estimate for the relative displacements at 0·4° since the $\Delta H'$ values are all small).

The electrostatic contribution to ($\Delta G_{III}^\circ - \Delta G_{IV}^\circ$) is thus only of minor importance in 0·05M-sodium chloride, although at lower salt concentrations tentative calculations indicate that (i) and (ii) may be of comparable magnitude and opposite in effect. Under the conditions of this study, it therefore appears that ($\Delta G_{III}^\circ - \Delta G_{IV}^\circ$) is determined mainly by (i) and so by the differences in free energy of formation of the two types of hydrogen bonds in eqns. (3) and (4). The observed value of +200 to +480 cal./mole for this quantity is reasonable since the free energy of formation of a hydrogen bond is of the order of only a few thousand calories. The positive sign indicates that the specific hydrogen bonds [eqn. (4)] in the double-helical form are, not surprisingly, more stable than those involving solvent molecules [eqn. (3)] in the denatured form.

It has been concluded,[1,4] and is assumed here, that the reversible back-titration curve at 25° of denatured herring-sperm deoxyribonucleate in 0·05M-sodium chloride below pH 8 can be accounted as the summation of the dissociations of primary phosphoryl groups (pK_a ~1—2), of guanine 2-amino-groups ($pK_a' = 2·75$),* of adenine 6-amino-groups ($pK_a' = 3·5$),[1] and of cytosine (and 5-methylcytosine) 6-amino-groups ($pK_a' = 5·0$).[1,8] Spreading of the dissociation ranges by polyelectrolyte effects is small and there is no need to postulate any significant quantities of secondary phosphoryl groups.[1] Since 80% of a dissociation occurs in the range pH = $pK_a' \pm 1$, then, on the basis of the pK_a' values quoted, the amino-groups of guanine and adenine should overlap at pH below 3·75 and of adenine and cytosine at pH 4·0—4·5. At pH 3·75—4·0 only the adenine amino-group should be titrated and at pH 4·5 to neutrality only the cytosine amino-group. Hence, as described by Wyman [2] for protein dissociations, $\Delta H'$ should vary with pH where groups overlap, *viz.*, below 3·75 and at 4·0—4·5, but at pH 3·75—4·0 and above 4·5, $\Delta H'$ should remain constant at the values characteristic of the 6-amino-groups of adenine and cytosine. These pH ranges are only approximate since they have been deduced from results at 25°.

* The pK_a' for the 2-amino-group of guanine has been obtained [9] by comparison of the back-titration curves of sodium deoxyribonucleate (kindly supplied by Dr. G. L. Brown of King's College, London) from *Arbacia lixula* sperm and from avian tubercle bacilli. These samples had very different guanine contents [10] (*viz.*, 17 and 36 moles of guanine/100 moles of nucleotides respectively).

[8] Hurlen, Laland, Cox, and Peacocke, *Acta Chem. Scand.*, 1956, **10**, 793.
[9] Cox, Thesis, Birmingham, 1955.
[10] Brown, M'Ewen, and Pratt, *Nature*, 1955, **176**, 161.

The $\Delta H'$ values obtained from the reversible back-titration curves (Fig. 3) and from Fig. 5 are listed in the Table. At pH 3·75—4·35 and possibly also at pH values above 5·5, $\Delta H'$ was constant within the allowed variation and this is in accord with the above predictions. The actual $\Delta H'$ values are of the same order as those deduced by Vandendriessche [6] for ribonucleic acid, namely, about +0·9—1·4 kcal./mole at pH 5·5—5·6 and +7·8—9·0 kcal./mole at pH 9·4—9·5, although his interpretation of the former as characteristic of secondary phosphoryl groups cannot be accepted for his undegraded sample, which contained only very few of these groups.

Interpretation of the variation of $\Delta H'$ *with* pH.

h range	pH range (approx.)	$\Delta H'$ (kcal./mole)	Groups assigned
>1·4	<3·75	Varying	Guanine 2-amino Adenine 6-amino
1·4—0·85	3·75—4·35	— 1·0	Adenine 6-amino
0·85—0·4	4·35—5·5	Varying	Adenine 6-amino Cytosine 6-amino
0·10—0·4	5·5—7·0	*ca.* + 2·0	Cytosine 6-amino
—	9·5—11·0	+ 6·0	Guanine and thymine 1 : 6 –NH·CO–

The authors thank Professor M. Stacey, F.R.S., for support and interest and the Department of Scientific and Industrial Research for a maintenance grant (to R. A. C.).

CHEMISTRY DEPARTMENT,
THE UNIVERSITY, BIRMINGHAM, 15.

[*Received, June 28th,* 1957.]