485. *Electrometric Titration of the Sodium Salts of Deoxyribonucleic Acids. Part III.* The Effect of Sodium Chloride.*

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The dissociation curves at controlled electrolyte concentrations of herring-sperm sodium deoxyribonucleate have been studied; cells without a liquid junction were used in a non-continuous method which avoids hydrolysis. The difference between the irreversible forward- and reversible back-titration curves was fully present at ionic strengths from 0·02 to 0·50 and shows that in solution the hydrogen bonding in the double-helical deoxyribonucleate structure is unimpaired by such changes of ionic strength. Under these experimental conditions, the two back-titration curves from pH 2·2 and 12 were exactly coincident, showing that hydrogen bonds involving titratable groups can link only the 1 : 6 amino- and –NH·CO– systems of the bases. The titration curves were displaced in a parallel manner with increasing salt concentration and this can be interpreted as indicating an exchange on the nucleate anion between sodium and hydrogen ions. The region of the back-titration curves from pH 6 to 8·5 is re-examined in view of the better values now available for base compositions and pK_a' values of the amino-groups and it is concluded that there are no secondary phosphoryl end groups in this preparation which are detectable by titration methods. Evidence is presented that solutions of the deoxyribonucleate can be slowly hydrolysed by alkali at pH 12 even at 25°. Improvements in the procedure for continuous titration of nucleates have been tested.

The study of sodium deoxyribonucleate has in recent years been greatly facilitated by the elucidation by *X*-ray diffraction studies of its molecular structure in the moist solid state.[1] It appears to consist of two intertwined helical polynucleotide chains inside which purine and pyrimidine rings, one from each chain, are cross-linked by hydrogen bonds.[2] The bases which can be so linked in this structure are the pairs adenine–thymine and guanine–cytosine. The hydrogen bonds, according to this model,[2] link the –NH·CO– groups at the 1 : 6-positions of thymine or guanine with the $-N{:}C(NH_2)-$ groups at the 1 : 6-positions of adenine or cytosine, respectively. The former groups (often referred to as the " enolic

* Parts I, II, *J.*, 1951, 3361, 3374.

[1] Watson and Crick, *Nature*, 1953, **171**, 737; Wilkins, Stokes, and Wilson, *ibid.*, p. 738; Franklin and Gosling, *ibid.*, 1953, **172**, 156.

[2] Watson and Crick, *ibid.*, 1953, **171**, 964.

hydroxyl" groups) titrate at pH's above 8 whereas the amino-groups of the latter system titrate at pH's below 7. It is because of this important structural rôle of hydrogen bonds between dissociable groups that the titration curves of these substances can be informative about their structure. Indeed the first evidence for the existence of hydrogen bonds of the type described above came from titration studies [3,4,5] which showed that the curves obtained when solutions of thymus deoxyribonucleate, initially at pH 6·5—7·0, were first titrated to pH 2·5 and pH 12 differed markedly from those obtained on back-titration with alkali and acid respectively.

In the original study by Gulland *et al.*[3] the points on the back-titration curves after acid treatment and after alkali treatment differed by about 0·1—0·2 equiv. per 4 g.-atoms of nucleate phosphorus and were regarded by them as coincident.* These authors titrated solutions which were prepared after drying the moist, solid nucleate over phosphoric oxide (110°; 30 min.) but subsequent work [5] showed that the difference between the two back-titration curves could not be ignored when the solutions titrated were prepared from nucleate not previously dried in this way. This apparent difference in titration behaviour between solutions prepared from dried and undried nucleate has also been obtained by Jordan and his co-workers [6] and has been tentatively explained [7] in terms of a slight pre-hydrolysis of the nucleate during drying at 110°. The occurrence of slow alkaline hydrolysis of internucleotide linkages during the usual process of continuous titration above pH 10 has been suggested [5] as one of the possible explanations of the incomplete coincidence of the back-titration curves from pH 2·2 and from pH 12. In a continuous titration, neither the time at both extremes of pH nor the concentration of electrolytes is precisely controlled, especially if a liquid junction and salt bridge are employed. One of the aims of the investigation here reported was to titrate herring-sperm sodium deoxyribonucleate in such a way that these two variables were respectively minimal and constant, in order to allow a more precise test of the coincidence of the two back-titration curves. If the curves proved to be *exactly* coincident, this would exclude various suggested [8,9] labile linkages involving the amino- and –NH·CO– groups and confirm hydrogen bonds between them as the only possibility.

In order to obtain results of the necessary precision, the points on the dissociation curves were obtained by a method [10] involving E.M.F. measurements on individual mixtures in a cell without liquid junction. This allowed exact control of the time each mixture was held at the extremes of pH, especially pH 12, and also enabled titration curves to be obtained at various ionic strengths without the usual difficulties of comparison which arise from the inclusion in the measured pH's of an unknown variable liquid-junction potential effect. This technique also enabled a study to be made of the alkaline hydrolysis of the nucleate which is relevant to the possible existence of triesterified phosphoric acid residues (branching points) in nucleic acids.[11] A knowledge of such errors is particularly important in trying to determine the proportion of secondary phosphoryl end groups from titration curves.[11] Finally, the effect of changes in ionic strength on the titration curves of nucleic acids may be compared with their effects on the titration curves of proteins and of synthetic polybasic acids. Such comparisons should be helpful in formulating suitable models for the electrostatic behaviour of the nucleic acid molecule.

The Titration Curves.—The titration curves of herring-sperm sodium deoxyribonucleate

* Here, and in what follows, "acid treatment" and "alkali treatment" refer, unless otherwise stated, to titration at 25° with acid to about pH 2·2 and with alkali to pH 12, respectively, immediately followed by back-titration with alkali or acid.

[3] (*a*) Gulland, Jordan, and Taylor, *J.*, 1947, 1131; (*b*) Cosgrove and Jordan, *J.*, 1949, 1413.
[4] Signer and Schwander, *Helv. Chim. Acta*, 1949, **32**, 853.
[5] Lee and Peacocke, *J.*, 1951, 3361.
[6] Garner, Jordan, and Matty, quoted by Jordan in "The Nucleic Acids," ed. Chargaff and Davidson, Academic Press Inc., New York, 1955, p. 477.
[7] Peacocke, *Biochim. Biophys. Acta*, 1954, **14**, 157.
[8] Euler and Fono, *Arkiv Kemi, Mineralog., Geol.*, 1948, **25**, No. 3; Little and Butler, *J. Biol. Chem.*, 1951, **188**, 695.
[9] Cavalieri and Angelos, *J. Amer. Chem. Soc.*, 1950, **72**, 4686.
[10] Lee and Peacocke, *Research*, 1953, **6**, 15s.
[11] Peacocke, Proc. Intern. Symp. Macromol. Chem., Milan–Turin (Sept. 1954), *Ricerca sci.*, 1955, **25**.

(at constant concentration) were obtained at 25°, while the ionic strength (μ) due to all ions other than those from the nucleate was kept constant. In practice this meant that, below pH 7, the sum {[NaCl] + [HCl]} and, above pH 7, the sum {[NaCl] + [NaOH]} were kept constant for each titration curve at the values of 0·02, 0·05, 0·15, and 0·50, severally. Even in the most acidic and most alkaline solutions the ionic strength was almost entirely due to the sodium chloride, whereas the contribution to the ionic strength of the sodium ions from the nucleate was only of the order of 0·005. The total concentration of sodium ions was exactly constant for a given curve in alkaline solution but, owing to the nature of the mixtures, it varied by up to 0·007M in acidic solutions. The curves obtained at the various non-nucleate ionic strengths (μ) are given in Figs. 1—4.

At ionic strengths of 0·02 and 0·05 (Figs. 1 and 2) the forward-titration curves with alkali to pH 12 (I) and with acid to pH 2·2 (III) were both different from that obtained (II) on back-titration from these extremes of pH. Points which represented the result of back-titration with alkali from pH 2·2 or with acid from pH 12 both lay on the same curve (II) to a high degree of precision. The reversibility of the dissociations represented by curve (II) was demonstrated by back-titration of mixtures with alkali from pH 2·2 to pH 12 followed by a second back-titration with acid or *vice versa*. The resulting points also fell accurately on line II. At these ionic strengths it appeared to matter little in the acid branches of the titration curves whether or not sodium chloride was added to an individual titration mixture before or after the addition of acid. Fig. 2 also shows the back-titration curve of acid-treated nucleate obtained by several independent continuous titrations of single nucleate solutions containing sodium chloride (0·05M), a glass electrode and a cell with liquid junction being used. The pH scale of this curve differs from that employed for the rest of the diagram so that it is slightly displaced along the pH axis (and in opposite senses above and below pH 7). However it lies exactly parallel to the more accurate curve II and this shows that, apart from the difficulties inherent in this method at alkaline pH's, continuous curves can be obtained reproducibly with the correct shape and curvature. The different relative sign of the displacement above and below pH 7 is probably related to a change in the liquid-junction potential in the glass electrode–calomel electrode system in passing from acid to alkaline solution.

At ionic strengths of 0·15 and 0·50 (Figs. 3 and 4) curves I and II were similarly disposed with respect to each other, but a more complex pattern occurred in the acid region. When sufficient acid was added to nucleate solutions in the absence of sodium chloride in order to lower the pH to 2·2 and was then followed by the addition of alkali (for back-titration) and sodium chloride, the resulting solutions yielded points (open circles) on the curve (II) corresponding to back-titration from pH 12. Only in this way could there be measured the effect of sodium chloride on the back-titration curve of a nucleate devoid of hydrogen bonds after acid treatment. For when salt was added first, the forward-titration curve III was obtained with acid and on back-titration with alkali gave curve IV. Precipitation intervened at higher pH values in these larger sodium chloride concentrations so that the forward-titration had to stop before all the relevant amino-groups were ionised and before all hydrogen bonds had been ruptured—as proved by the continuation of IV (not shown in Fig. 4) into the alkaline region, where it lies between I and II and by a comparison of IV with II. The displacement of the titration curves to lower pH values with increasing sodium chloride concentration (Figs. 5 and 6) also enhanced this effect. No such complication occurred with the forward-titration to pH 12 since there was no question then of precipitation and complete rupture of all the hydrogen bonds could be attained even more readily when salt was present. As at the lower ionic strengths, curve II could be demonstrated to represent a reversible dissociation process. At all ionic strengths from 0·02 to 0·50 variation of the concentration of the sodium nucleate within the limits 0·07—0·21 mg. of phosphorus per ml. did not affect the positions of the titration curves.

The reversible titration curves (II, Figs. 1—4) of the nucleate containing no hydrogen bonds were displaced to lower pH values with increasing ionic strength (Fig. 5). This displacement was parallel with respect to pH and was almost the same for the acid (pH < 6) and alkaline (pH > 9) branches except that in the latter the displacement reached a limiting value between $\mu = 0{\cdot}15$ and 0·50 since the curves at these two ionic strengths

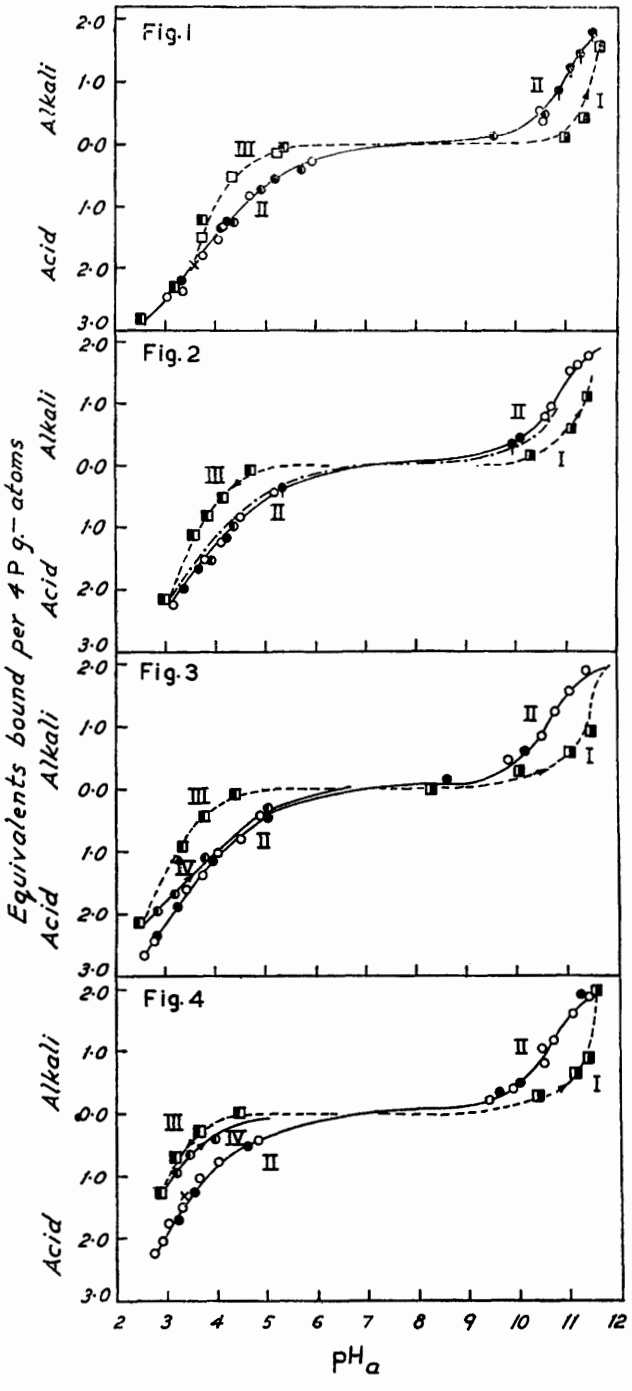

FIG. 1. *Titration curves of sodium deoxyribonucleate, at* 25°, $\mu = 0{\cdot}02$.

Acid treatment.

Salt added at pH 6—7 before acid/alkali:

Forward-titration with acid to pH 2·2 . . . . . ◧

Back-titration with alkali from pH 2·2 . . . . . ◐

Acid/alkali added before salt:

Forward-titration with acid to pH 2·2 . . . . . □

Back-titration with alkali from pH 2·2 . . . . . ○

Alkali treatment.

Salt present, order of addition found not to be important.

Forward-titration with alkali to pH 12 . . . . . ◨

Back-titration with acid from pH 12 . . . . . ●

Coincident ○ and ● points represented by . . . . . ◉

Reversibility test: Acid added to pH 2·2, followed by alkali and then acid; or alkali to pH 12, followed by acid and then alkali . . ×

Broken lines are forward-titration curves from pH 6—7; solid lines are back-titration curves.

pH$_a$ scale, see Experimental section.

FIG. 2. *Titration curves of sodium deoxyribonucleate, at* 25°, $\mu = 0{\cdot}05$.

As for Fig. 1.

—·—·— Back titration curve with alkali from pH 2·5 at $\mu = 0{\cdot}05$ obtained by the continuous titration method, with a glass electrode in a cell with liquid junction (pH scale for this curve, see Experimental section).

FIG. 3. *Titration curves of sodium deoxyribonucleate, at* 25°, $\mu = 0{\cdot}15$.

As for Fig. 1.

FIG. 4. *Titration curves of sodium deoxyribonucleate, at* 25°, $\mu = 0{\cdot}50$.

As for Fig. 1.

Note.—The size of the symbols in these and Figs. 6 and 7 do not represent experimental error.

were very close.* There was a slight deviation from this parallel arrangement close to the isoelectric point and this can be seen most clearly around pH 3·8 at $\mu = 0{\cdot}50$ (Fig. 5) when the isoelectric point was relatively high. The forward-titration curves were also displaced in a parallel manner with increasing μ and there was a marked difference between the displacement of the acid and alkaline branches (Fig. 6).

Since a known controlled chloride concentration was necessary for measurements with the cell without liquid junction, titration curves at very low electrolyte concentrations were obtained with the glass–calomel electrode cell. Fig. 7 shows the forward and back

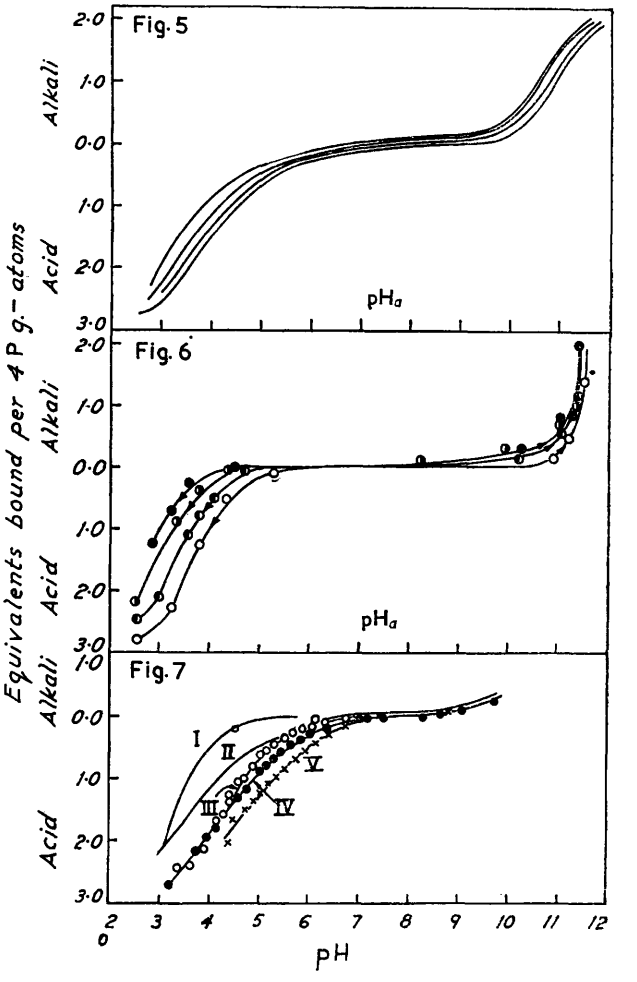

FIG. 5. *Reversible back-titration curves of sodium deoxyribonucleate at various μ.*

Curves at $\mu = 0{\cdot}02$, 0·05, 0·15, 0·50, reading from right to left.

FIG. 6. *Forward-titration curves of sodium deoxyribonucleate at various μ.*

Curves at $\mu = 0{\cdot}02$, 0·05, 0·15, 0·50, reading from right to left.

FIG. 7. *Titration curves of sodium deoxyribonucleate at low μ.*

All curves obtained by continuous method with glass electrode and in a cell with liquid junction.

I, II Forward- and back-titration curves, to and from pH 2·5, $\mu = 0{\cdot}05$.

III, IV Forward- (○) and back-titration curves (●), to and from pH 2·5, with no salt added, $\mu \leqslant 0{\cdot}007$.

V (×) Reversible titration curve with acid of deoxyribonucleate previously acid-treated (to pH 2·2) neutralised and dialysed.

pH scale, see Experimental section.

continuous titration curves of the nucleate without added sodium chloride (III, IV) and the reversible " back "-titration curve (V) of a sample of the nucleate which had previously been acidified to pH 2·2, neutralised to pH 7, and dialysed to remove the small amount of salt resulting from this procedure. A small amount of electrolyte ($\mu \gtrsim 0{\cdot}007$M) is inevitably present in the titration represented by IV and even less in that represented by V. The forward- and back-titration curves (I, II) obtained by continuous titration in the presence of 0·05M-sodium chloride are also given for comparison. It is clear that, although the precise ionic strengths along IV and V are not known, the reversible back-titration

* The portion of the curves between pH 6 and 9 on Figs. 1—5 is conjectural since stable E.M.F. readings cannot be obtained with the hydrogen electrode in this range.

curves were very sensitive to small changes in μ when this was low and that the displacement was still approximately parallel.

In cases where precipitation did not intervene (*i.e.*, $\mu \leqslant 0{\cdot}05$) the meeting point of the forward-titration curve with acid and the back-titration curve with alkali represented the minimum amount of acid that had to be bound to ensure the irreversible rupture of all hydrogen bonds. This quantity decreases as the sodium chloride concentration was lowered, having the values 2·3, 1·9, and 1·5 equivs./4 P g.-atoms for the curves at $\mu = 0{\cdot}05$ (II, III, Fig. 2), $\mu = 0{\cdot}02$ (II, III, Fig. 1), and $\mu \simeq 0{\cdot}007$ (III, IV, Fig. 7) respectively.

Alkaline Hydrolysis at 25°.—The pH of a solution of sodium nucleate (final $\mu = 0{\cdot}02$) was adjusted to 12 by the addition of alkali and, after varying intervals, sufficient acid was added to lower the pH to about 4—5 or 10. The usual E.M.F. readings on these and the appropriate blank solutions then gave the equivalents of alkali that had disappeared from the solution by combination with the dissociable groups of the nucleic acid and by being used up in any hydrolytic reaction. The former could be obtained at any particular final pH from curve II of Fig. 1, hence the amount used up in combination with groups released by any hydrolysis could be computed with a maximum error of about $\pm 0{\cdot}03$ per 4 P g.-atoms. An increasing uptake of alkali due to this latter cause was detected when the final pH was about 10 but not when it was about 4 or 5 (Table 1). This implies that alkali at pH 12 did slowly hydrolyse the nucleate at 25° and that it released groups which titrated somewhere between pH 5 and 10.

Discussion

(a) *Hydrogen Bonding.*—The present titration studies show that when the ionic strength is maintained constant and the time the nucleate is kept at extreme pH's is reduced to a minimum, the back-titration curves after mild acid- and alkali-treatment are exactly coincident at all the ionic strengths studied (Figs. 1—4). The difference between these two back-titration curves observed [3, 5, 6] in continuous titrations must therefore arise from causes specific to that method and will be discussed below. It follows from this exact coincidence of the back-titration curves that the forward-titration with acid of the amino-groups effects exactly the same changes in the nucleate as the forward-titration with alkali of the –NH–CO– systems. During both these forward-titrations from neutrality the groups

Table 1. *Alkali bound by sodium deoxyribonucleate (concn.* = 0·141 *mg.* P/*ml.) due to hydrolysis at* pH 12 *and* 25°.

Time of treatment with alkali (min.)	E.M.F. of final mixture (E_{DNA}) (mv)	Equivs. of acid added	Final pH	Equivs. of alkali bound/4 P g.-atoms: Total	By DNA at t = 0·5 min.*	By groups released by hydrolysis
0·5	922·0	18·76	10·32	0·30	0·30	0·0
67	920·0	—	10·29	0·33	0·24	0·09
102	912·5	—	10·13	0·36	0·26	0·10
170	914·0	—	10·20	0·34	0·26	0·08
822	897·5	—	9·90	0·41	0·16	0·25
0·5	579·0	29·86	4·11	1·23	1·23	0·0
780	583·2	—	4·11	1·23	1·23	0·0
0·5	640·3	27·41	5·10	0·52	0·52	0·0
1020	641·7	—	5·10	0·52	0·52	0·0

* From Curve II, Fig. 1.

become ionised and the hydrogen bonds in which they were involved disappear. This rupture of hydrogen bonds appears to be irreversible when a sufficient number of adjacent bonds have been broken to cause irreversible changes in the configuration of the polynucleotide chains.* The exactness of the coincidence of the two back-titration curves after this

* The processes occurring along the forward-titration curve have been studied by graded titration to and from various pH's and by other methods.[12]

[12] Cox and Peacocke, Proc. Intern. Symp. Macromol. Chem., Israel (April, 1956), *J. Polymer Sci.*, in the press.

process means that hydrogen bonds involving groups titratable between pH 2·5 and 12 must link only the amino-groups ($pK_a' < 7$) with the –NH·CO– groups of guanine and thymine ($pK_a' > 7$). Any other bonds [8,9] in the original deoxyribonucleate which involve these groups are thereby excluded. This is in accordance with the double-helical hydrogen-bonded structure postulated for the moist solid state [1,2] and indicates strongly that this structure exists also in solution. Changes in ionic strength over a wide range do not decrease the extent of hydrogen bonding as shown by the persisting large difference between curves I and II at all the ionic strengths of Figs. 1—4. Clearly, changes in viscosity observed on the addition of sodium chloride cannot be directly attributed to rupture of hydrogen bonds.

As the pH decreases in the forward-titration, the amino-groups must titrate in the order cytosine, adenine, and guanine, judging from their known pK_a' values. According to the double-helical structure the 2-amino-group of guanine is not hydrogen-bonded and this is not inconsistent with the observations. The minimum number of groups that have to be ionised before all the hydrogen bonds are irreversibly ruptured (" denaturation ") decreases as μ decreases from 0·05 and eventually falls to a value, 1·5, lower than the total number of adenine and cytosine amino-groups present (2·0/4 P atoms). It is clearly not always necessary to ionise all the amino-groups involved in hydrogen bonds in order to denature the whole molecule. Ionisation of the amino-groups and the usual thermal effects must therefore be regarded as jointly operative in denaturation.[12] As μ decreases from 0·05, less ionisation appears to be necessary to achieve complete denaturation and similar " protective " effects of salt have been reported.[13] They may arise from an increased accessibility of the inside of the double helix or from a weakening of the phosphoester linkages [13b] when the helical structure is expanded by the increased repulsion between primary phosphate charges at the lower μ.

When μ increases to values above 0·05, the shift of the titration curves and earlier precipitation can make it impossible to attain the minimum amount of ionisation necessary for complete rupture of all hydrogen bonds by acid (*e.g.*, III, IV, Figs. 3, 4). Such effects should be taken into account when, for example, the effect of acid treatment of sodium deoxyribonucleate on its light-scattering behaviour is being studied. In such investigations, in order to obtain the completely acid-denatured nucleate, the acid (and any neutralising alkali) must be added *before* the addition of the sodium chloride necessary for the light-scattering measurements, if this exceeds 0·05M. Some of the discrepancies in the results of various light-scattering studies [14] can be attributed to this cause.

(b) *Continuous Titration Curves.*—In a previous study [5] solutions of sodium deoxyribonucleate were titrated continuously in cells with liquid junction and without addition of sodium chloride to give the complete forward- and back-titration curves. Under these conditions the back-titration curves were found to differ appreciably and this observation has been confirmed by others.[6] The explanations originally advanced [5] for this can now be re-examined. (i) The sensitivity of the back-titration curves to salt is greatest at low salt concentrations (Fig. 7), so that even slight diffusion from the salt bridge into the salt-free solution could have a detectable effect. (ii) The presence of hydrogen bonds broken only by acid- or only by alkali-treatment was a possible explanation, but is now excluded in view of the preceding discussion. (iii) Alkaline hydrolysis was considered to be the most likely explanation of the difference, and Table 1 shows that under continuous-titration conditions (25°, at pH 12 for up to 30 min. and at pH 10·5—12 for 1—2 hr.) alkaline hydrolysis could account for up to 0·1 equivalent of alkali combined per 4 phosphorus g.-atoms along the back-titration curve after alkali treatment. The smaller difference between the continuous back-titration curves when the nucleate had previously been dried at 110° could then be understood in terms of a slight pre-hydrolysis, during drying, of the bonds first attacked by alkali.[7] Even after allowance for the operation of factors (i) and (iii) there still seems to be a residual difference in the continuous back-titration curves of about 0·2—0·3 equivalent above pH 7. Separate experiments have now shown that even

[13] (*a*) Thomas, *Biochim. Biophys. Acta*, 1954, **14**, 231; (*b*) Doty, 3rd Intern. Congr. Biochem., Brussels, Aug. 1955; (*c*) Sadron, as (*b*).

[14] (*a*) Reichman, Bunce, and Doty, *J. Polymer Sci.*, 1953, **10**, 109; (*b*) Horn, Leray, Pouyet, and Sadron, *ibid.*, 1952, **9**, 531; (*c*) Alexander and Stacey, *Biochem. J.*, 1955, **60**, 194.

at 25° the attack of alkali on Pyrex glass during the time of a lengthy continuous forward- and back-titration of a nucleate solution can be sufficiently great to account for this.

The above conclusions indicate that in order to obtain useful results from continuous titrations of nucleates in cells with liquid junctions the following additional precautions should be observed: control of ionic strength by the presence of salt, to prevent (i); and avoidance of long periods of alkaline pH which cause (iii) and the attack on glass. In practice, the first condition is fulfilled by the presence of 0·05M-salt when the sensitivity to further additions of salt is small (Fig. 5) and when the addition of acid to pH 2·5 breaks all hydrogen bonds, even though the salt is present (Fig. 2). The second condition is best fulfilled by confining continuous titrations to forward-titration with acid followed by back-titration with alkali, the latter being executed as quickly as possible once the pH rises above 10·5. With these precautions, the standard deviation of the curves (25°, $\mu = 0{\cdot}05$; I, II, Fig. 7) was only 0·02—0·04 equivalent per 4 g.-atoms of phosphorus at pH 3—11, and the mean curve was parallel to that obtained by the more rigorous non-continuous technique (see Fig. 2). The continuous back-titration curves obtained by this method after acid- and alkali-treatment were coincident below pH 7, but still differed in the same direction as before [5,6] above pH 7, chiefly owing to the effect of glass during alkali-treatment. These results define the present range of usefulness for nucleates of the much more convenient and economical continuous titration method, which, when a glass electrode is used, also has the advantage of giving steady readings in the range pH 5—9.

(c) *Effect of Sodium Chloride.*—The groups in the completely non-hydrogen-bonded sodium nucleate dissociate freely and reversibly, and Figs. 5 and 7 therefore demonstrate the effect on these free dissociations of increasing the non-nucleate ionic strength (μ) which in the present instance has the same numerical value as the concentration of sodium ions. The back-titration curves are displaced in a parallel manner to lower pH values, except at pH's approaching the isoelectric point appropriate to each μ. A similar parallel shift has been reported for the effect of sodium chloride on the spectrophotometric titration curves of thymus sodium deoxyribonucleate.[15] If the effective charge on the nucleate anion were compounded only of the charges on the dissociable groups then it would be expected that the displacement of the curves caused by a given increase in μ would be greater the more alkali that was bound, as with globular proteins [16] and with polybasic acids.[17] Thus the total *effective* charge on the nucleate anion must include other factors besides the charge on the dissociable groups. Evidence for the binding of other ions has been obtained by Shack *et al.*[18] who showed that at neutral pH thymus sodium deoxyribonucleate binds about $2{\cdot}3 \pm 0{\cdot}3$ g.-ions of sodium and $0{\cdot}3 \pm 0{\cdot}1$ g.-ions of chloride per 4 P g.-atoms from sodium chloride solutions stronger than 0·005M.

The curves obtained on titration of insoluble fibrous proteins, such as wool,[19] with hydrochloric acid and sodium hydroxide also undergo a parallel shift with increasing sodium chloride concentration. It appears that as each hydrogen ion is bound a chloride ion also enters the fibre [20] and that when a hydroxyl ion is bound (*i.e.*, a hydrogen ion is dissociated) a sodium ion enters as a result of the high potential acquired by the fibre as a whole when the fractional charge due to the dissociating groups is still relatively small. When negative, this high potential prohibits the further dissociation of hydrogen ions unless sodium ions enter at the same time. The various theoretical treatments of this system differ according to whether the ions (Cl^- or Na^+) accompanying the hydrogen or hydroxyl ions are regarded as bound to specific sites [20a] or as simply dissolved in the imbibed water within the fibre.[20b] The first hypothesis is probably more useful for the nucleate anion which is known to bind sodium ions,[18] presumably on the primary phosphoryl charges.

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

[16] (*a*) Cannan, Kibrick, and Palmer, *Ann. New York Acad. Sci.*, 1941, **41**, 243; (*b*) Tanford, *J. Amer. Chem. Soc.*, 1950, **72**, 441; (*c*) Scatchard, *Ann. New York Acad. Sci.*, 1949, **51**, 660; (*d*) Cohn and Edsall, "Proteins, Amino-acids and Peptides," Reinhold Publ. Corp., New York, 1943, pp. 468 *et seq.*

[17] Katchalsky, Shavit, and Eisenberg, *J. Polymer Sci.*, 1954, **13**, 69.

[18] Shack, Jenkins, and Thompsett, *J. Biol. Chem.*, 1952, **198**, 85.

[19] Steinhardt and Harris, *J. Res. Nat. Bur. Stand.*, 1940, **24**, 335.

[20] (*a*) Gilbert and Rideal, *Proc. Roy. Soc.*, 1944, *A*, **182**, 335; (*b*) Vickerstaff, "The Physical Chemistry of Dyeing," Oliver and Boyd, London, 1954, pp. 351 *et seq.*

The titration curves of a cross-linked poly(methacrylic acid) ion exchange resin are also displaced in a parallel manner with increasing salt concentration [21] and in this case sodium ions undoubtedly exchange with hydrogen ions (cf. the hydrogen-ion dissociation of some polyelectrolyte gels [22]).

Hence the parallel displacement of the nucleate titration curves with increasing concentration of sodium chloride suggests that sodium ions enter the nucleate anion to replace hydrogen ions that are dissociated. A similar conclusion [23] has also been reached concerning the effect of sodium chloride on the binding of a small organic cation, proflavine, by sodium deoxyribonucleate. Application of a thermodynamic approach [20a] to such exchange of sodium and hydrogen ions by a molecule containing only one type of site binding each ion leads to the following relation :

$$\mathrm{pH} = -\log[\mathrm{Na^+}] + 2\log\left(\frac{\alpha}{1-\alpha}\right) + \frac{2}{2{\cdot}303RT}(\Delta\mu_{\mathrm{Na+}} - \Delta\mu_{\mathrm{H+}}) \quad . \quad . \quad (1)$$

where α is the degree of dissociation of the group binding hydrogen ions and $\Delta\mu_{\mathrm{Na+}}$, $\Delta\mu_{\mathrm{H+}}$ are the changes in standard chemical potential of the two ions on being bound. At constant α, a plot of $(\mathrm{pH})_\alpha$ against $-\log[\mathrm{Na^+}]$ should be linear with a slope of $+1$. The same relation should hold even for titration curves in which the dissociation of various groups overlap, as with the nucleic acids. Fig. 8 shows that for the herring-sperm deoxyribonucleate this plot approximates to a straight line of slope $+0{\cdot}5$ the only significant deviation occurring with the alkaline branch when μ is more than 0·15. With fibrous proteins,[19] a polymethacrylic ion-exchange resin,[21] and myosin solutions [24] the corresponding plots were lines of respective slopes of $+1$, $+1$, and $-0{\cdot}5$ (difference in sign because positively charged myosin being considered). The deviation of the slope of the line in Fig. 8 from unity could be attributed to the binding of ions other than sodium (*e.g.*, chloride [18]), which would modify the simplified derivation of eqn. (1).

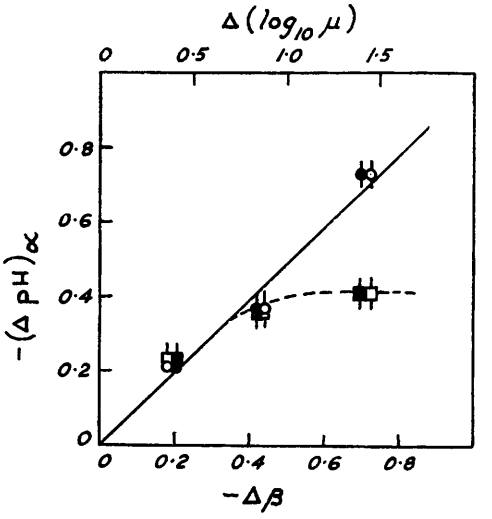

FIG. 8. *Relation between* pH *displacement of the back-titration curves and* μ.

Ordinate : ΔpH = Average increase in pH along the various back-titration curves relative to the $\mu = 0{\cdot}02$ curve (II, Fig. 1).

Abscissa : Upper scale, $\Delta(\log_{10}\mu) = \log_{10}\mu - \log_{10}0{\cdot}02$.
Lower scale, $\Delta\beta = \beta_\mu - \beta_{\mu=0{\cdot}02}$.

Definition of β, see ref. 25, eqn. (9).

○ ΔpH *v.* $\Delta\beta$, pH <7.
□ ,, ,, pH >7.
● ΔpH *v.* $\Delta(\log_{10}\mu)$, pH <7.
■ ,, ,, pH >7.

An alternative approach to the salt effect is possible if one assumes that the sodium ion is completely dissociated throughout and that increasing the sodium chloride concentration merely causes greater shielding by the ionic atmosphere around the nucleate anion. If the macro-ions are assumed to be spherical, this approach [16] requires as result a linear dependence of $(\mathrm{pH})_\alpha$ on $\mu^{\frac{1}{2}}$, but the present data yield a curved plot even at low μ. Hill has recently [25] calculated the electrostatic free energy, in solution, of a long cylinder

[21] Hale and Reichenberg, *Discuss. Faraday Soc.*, 1949, **7**, 82.
[22] Katchalsky and Michaeli, quoted by Katchalsky, *Progr. Biophys.*, 1954, **4**, 1.
[23] Peacocke and Skerrett, *Trans. Faraday Soc.*, 1956, **52**, 261.
[24] Mihalyi, *Enzymologia*, 1950—51, **14**, 224.
[25] Hill, *Arch. Biochem. Biophys.*, 1955, **57**, 229.

uniformly charged on its surface, and application to his equation of the methods used for the spherical case [16] finally yields for the dissociation of one type of group:

$$\mathrm{pH} = \mathrm{p}K_0' + \log\left(\frac{\alpha}{1-\alpha}\right) - \left(\frac{2}{2{\cdot}303}\cdot\frac{N\varepsilon^2}{RTD}\right)\left(\frac{Z}{L}\right)\beta \quad . \quad . \quad . \quad (2)$$

where $\mathrm{p}K_0'$ = the apparent acidic dissociation constant of the groups when $Z = 0$, α = their degree of dissociation, Z = the total charge on the long cylinder of length L, and β is a function of ionic strength and the radii of the cylindrical and small ions [ref. 25, eqn. (9), quantity in square brackets]. Even if various dissociable groups are present whose titration ranges overlap, the change $(\Delta\mathrm{pH})_\alpha$ due to a change in salt concentration at constant hydrogen ions bound should be given by:

$$(\Delta\mathrm{pH})_\alpha = -\left(\frac{2N\varepsilon^2}{2{\cdot}303RTD}\right)\left(\frac{Z}{L}\right)\Delta\beta \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

The function β has been calculated for the present conditions and is found at first to fall very steeply with increasing salt concentration and then to change only very slowly above $\mu = 0{\cdot}1$. A plot (Fig. 8) of $(\Delta\mathrm{pH})_\alpha$ against $\Delta\beta$, taking the $\mu = 0{\cdot}02$ curve as the reference line, is as linear as the logarithmic plot already described. However, this linearity may be fortuitous since, over the range $\mu = 0{\cdot}02$—$0{\cdot}50$, β is very nearly a linear function of $\log \mu$. The strongest argument against this second approach is that equation (3) predicts an increase in $(\Delta\mathrm{pH})_\alpha$ as Z, the charge due to the dissociable groups, increases so that it cannot explain the parallel displacement with salt in Figs. 5 and 6. The parallel displacement could only be explained by (3), and by other equations similarly based on the assumption of a screening effect of the salt, if the molecule extended as Z increased so that Z/L remained approximately constant. During the back tititration of deoxyribonucleate from pH 3·1 to pH 11·5, Z changes four-fold, but there is little evidence about the dimensions of the molecule over this range, although some extension has been reported.[14c] Precise dimensional information is therefore required in order to decide if the effect of salt can be regarded as entirely due to its screening effects.

The experimentally observed displacements show that the overall negative charge density and potential on the complex of nucleate anion + gegenions can remain fairly constant over a wide range of pH, salt concentration, and charge on the polynucleotide chains, but under extreme conditions it appears to alter, for example, when: (*a*) the polynucleotide charge itself becomes small, as expressed in the deviation from parallelism of the curve at $\mu = 0{\cdot}50$ at low pH near the isoelectric point (Fig. 5), when the ion exchange character of the nucleate anion must be breaking down; and (*b*) both the polynucleotide charge becomes very negative and the salt concentration very high, as in the alkaline branch of the back-titration curve at $\mu > 0{\cdot}15$. Figs. 5 and 8 show that with increasing μ up to 0·15 this part of the curve is displaced to much the same extent as the acid branch but that beyond this point sodium chloride has little more effect. This implies that there is a limit to the number of sodium ions that can be accommodated around the polynucleotide chains and that this limit is attained when the chains have a very negative charge and when $\mu > 0{\cdot}15$. When the polynucleotide charge is less negative, as along the acid branch of the back-titration curve, this limit is not reached even at $\mu = 0{\cdot}50$.

(d) *Secondary Phosphoryl Groups.*—Estimation of the number of secondary phosphoryl groups in sodium deoxyribonucleates is important since they only occur on the monoesterified phosphoric acid residues at the ends of polynucleotide chains. Their proportion is therefore related to the degree of branching [11] or to the presence of an "interrupted" double helix.[26] They titrate with $\mathrm{p}K_a'$ about 6—7 and attempts have frequently been made [3, 5, 11] to estimate them from titration curves. When chromatographic analyses of the base composition became available, the procedure was [5] to sum the titration curves of each group, assuming free monobasic acid behaviour, and to use the analytical amounts of the bases and approximately the $\mathrm{p}K_a'$ values prevailing in the nucleotides. The latter values

[26] Dekker and Schachman, *Proc. Nat. Acad. Sci.*, 1954, **40**, 894.

were adjusted to give the summed theoretical curves best fitting the observed back-titration curves of the various nucleates. It was usually found that the region of the titration curves from pH 6 to 8·5 could not be satisfactorily accounted for by the amino- and –NH·CO– groups alone so that small but finite amounts of secondary phosphoryl groups (*ca.* 0·2—0·4 per 4 P atoms) were postulated.[5,27] Recent advances [28,29] in the hydrolytic procedures that precede chromatographic analysis yield results in which there is now almost complete recovery of the bases with respect to phosphorus (*e.g.*, the figures quoted in the Experimental section). Recently [30] the pK_a' of the cytosine amino-group has been found to be 5·0 (at $\mu = 0{\cdot}05$) in a nucleic acid derivative (reduced apurinic acid) from which the purines have been removed and for which a better estimate of this quantity is possible. This is higher than would have been expected from its value in the deoxyribonucleotide,[31] *viz.*, 4·6 in the absence of added salt. If this pK_a' value of 5·0 at $\mu = 0{\cdot}05$ also applies to the nucleate—and the structure of the reduced apurinic acid is closer to that of the nucleate than is that of a nucleotide—then it must be concluded that the cytosine amino-group dissociates over the range previously assigned to the secondary phosphoryl groups. The whole range of the back-titration curve obtained by the continuous method at $\mu = 0{\cdot}05$ (Fig. 2) can in fact be fitted from pH 4 to 8 by taking the analytical figures (see Experimental section) for the amounts of cytosine, including 5-methylcytosine, and adenine and assigning pK_a' values of 5·0 and 3·5 to their respective amino-groups. Doty and Rice [32] have suggested that the spread of the pK_a' of the cytosine amino-group due to polyelectrolyte charge effects would account for the back-titration curve over the range pH 6—8·5. Such effects may be partially responsible, although the change with pH of the pK_a' of the cytosine amino-group in reduced apurinic acid was much smaller than that observed with polyelectrolytes (it varies [33] by no more than 0·25 unit for pH 3·5—6·5). Whatever the explanation, the experimental evidence now suggests that the cytosine amino-group could be dissociating over pH 5—7 and this renders the previous estimates of secondary phosphoryl groups unnecessarily high. The evidence for a significant amount of such end groups based on dye-binding studies [9] has also been found capable of other interpretations.[11,23] Hence if branching does occur, it must be at a frequency too low for unambiguous detection by titration, and other more sensitive methods of investigation are necessary for this purpose.

(e) *Alkaline Hydrolysis.*—The earlier evidence that alkali at pH 11—12, even at 25°, slowly hydrolyses sodium deoxyribonucleate has been described in a previous paper. Since then Mathieson and Porter [34] report that they have obtained the " turbidimetric titration " curve of alkali-treated material and this may also be interpreted in terms of hydrolysis. Dialysable phosphorus-containing fragments have also been detected [35] after treatment with alkali at pH 12 for 24 hours at 37°, although Doty [13b] reports little change in the weight-average molecular weight after similar treatment. However the latter observation could be explained if the small dialysable fragments were obtained by rupture of linkages near to the chain ends. The results given in Table 1 show that alkali is slowly consumed in some reaction at pH 12 and 25°, and that it releases groups titrating between pH 5 and 10. Since the back-titration curves obtained in the absence of alkaline hydrolysis show that all the cytosine groups are free to titrate, these released groups can only be secondary phosphoryl released by hydrolytic cleavage of the diester linkages.

If there were present in the nucleate triesterified phosphoric acid residues, *i.e.*, branching points, then alkali would be expected to hydrolyse these linkages, with release of primary phosphoryl groups ($pK_a' \sim 1$—2), even more readily than it attacks the diester linkages in the rest of the main chain, in view of the instability of the known triesters of phosphoric

[27] Jordan, *Ann. Rev. Biochem.*, 1952, **21**, 233.
[28] Wyatt and Cohen, *Biochem. J.*, 1953, **55**, 774.
[29] Marsh, Thesis, Birmingham, 1954.
[30] Hurlen, Laland, Cox, and Peacocke, *Acta Chem. Scand.*, in the press.
[31] Hurst, Marko, and Butler, *J. Biol. Chem.*, 1953, **204**, 847.
[32] Doty and Rice, *Biochim. Biophys. Acta*, 1955, **16**, 446.
[33] Unpublished calculations on the data of ref. 30; Cox, Thesis, Birmingham, 1955.
[34] Mathieson and Porter, *Nature*, 1954, **173**, 1190.
[35] Tamm, Shapiro, Lipshitz, and Chargaff, *J. Biol. Chem.*, 1953, **203**, 673; Letham, Thesis, Birmingham, 1955.

acid to dilute alkali at moderate temperatures.[36] (There is however no direct evidence on phosphoric acid triesterified by 2-deoxyribose derivatives.) Table 1 shows that none of the groups released by alkali from the nucleate titrates below pH 5·1, so that no detectable quantities of new primary phosphoryl groups have been formed along with the secondary phosphoryl. These observations again indicate that no very large proportion of phosphoric acid residues can be triesterified. Nevertheless it must be emphasised that, even if there were branching points at 1 in every 20 phosphorus atoms, amounts of new primary phosphoryl groups which could be detected by the above methods would be released only if the rate of hydrolysis of the triesterified residue was many times greater than that of the diesterified residues.

Experimental

Materials.—Sodium deoxyribonucleate was isolated from herring sperm at 0—4° by the methods previously described.[37] The nucleoprotein was extracted by the method of Mirsky and Pollister,[38] and the protein separated by precipitation with excess of sodium chloride and centrifugation, followed by chloroform–octanol extraction of the last traces.[39] The sodium deoxyribonucleate was precipitated by ethanol, washed with ethanol and ether, dried in the frozen state, and stored as moist solid at room temperature. It had the following composition and characteristics: phosphorus = 9·22% (calc. 9·36%); Na/P (atomic) = 0·95; moles of base per 4 g.-atoms of phosphorus (chromatographic analyses by Dr. G. E. Marsh [29]), guanine 0·88, adenine 1·09, cytosine 0·81, 5-methylcytosine 0·07, thymine 1·08; $\lambda_{max.}$ 259 mμ ($\varepsilon_{(P)259}$ 7200), in the absence of salt. Light-scattering measurements in 0·1M-sodium chloride solution showed that this material had a molecular weight of $6·3 \times 10^6$ (Mr. B. N. Preston). This one preparation was used throughout the investigation and, for titrations, solutions of it in boiled distilled water were prepared. Nucleate concentrations were determined in terms of phosphorus which was estimated according to methods previously reported.[40]

Hydrochloric acid solutions were estimated gravimetrically as silver chloride and volumetrically by means of sodium borate decahydrate. Carbonate-free sodium hydroxide solutions were standardised against the hydrochloric acid in an atmosphere of hydrogen. Sodium chloride solutions were prepared from the dried " AnalaR " salt. Hydrogen from cylinders was passed over heated copper gauze to remove the last traces of oxygen, through concentrated sodium hydroxide to remove acidic gases, and finally through water at 25° to saturate it with water vapour before passage into the electrode cell.

Methods.—The hydrogen electrodes were prepared by platinising platinum foil (9 sq. mm.), spot welded to thin platinum wire which was fused into 3 mm. glass tubing. The silver–silver chloride electrodes were similar in construction except that platinum gauze replaced the platinum foil. The gauze was coated with silver oxide paste, baked at 450° for 3 hr., and was then made the anode for 20 sec. at a current of 20 mA in a cell containing hydrochloric acid (0·75M) and a platinum cathode. The electrodes were chosen for the rapidity with which they attained a steady potential in hydrochloric acid (0·01M), and all whose standard potentials lay outside the range 222·0—223·5 mv were rejected. Experiment showed that, although at sufficiently high concentrations nucleic acids may form precipitates with silver ions in solution, the silver–silver chloride electrodes gave the same potentials in chloride solutions of pH <10 before and after contact with nucleate. The potential difference between the pair of hydrogen electrodes used in the electrode vessel was never more than 0·1 mv, and the difference between the pair of silver–silver electrodes was never more than 0·50 mv.

The Pyrex-glass electrode vessel, which was immersed in a water-bath at 25° ± 0·05°, consisted of a horizontal portion (10 cm. × 8 mm. tubing) to which were attached six vertical arms (8 mm. in diameter), to accommodate the four electrodes and the hydrogen inlet and outlet. The cell was so placed that, when the hydrogen was bubbling through, the electrodes were intermittently washed by the solution (*ca.* 2 ml.). No other rocking device was found to be necessary. The nucleate solutions (final concentration 0·141 mg. of P/ml.), contained in wax-coated weighing bottles, were saturated with and stirred by the purified hydrogen stream during the addition of the reagents. For each mixture, acid (for final pH's down to 2·4) or

[36] Bailly and Gaumé, *Bull. Soc. chim. France*, 1936, **3**, 1396; Ross, *J.*, 1952, 4296.
[37] Laland, Lee, Overend, and Peacocke, *Biochim. Biophys. Acta*, 1954, **14**, 356.
[38] Mirsky and Pollister, *J. Gen. Physiol.*, 1946, **30**, 101.
[39] Sevag, Lackman, and Smolens, *J. Biol. Chem.*, 1938, **124**, 425.
[40] Jones, Lee, and Peacocke, *J.*, 1951, 623.

alkali (for pH's up to 12·0) was added and, within 30 sec., it was followed, if a point on the back-titration curve was being obtained, by the addition of the requisite volume of alkali or acid. Calculated volumes of sodium chloride solution and water were then added to bring the total non-nucleate ionic strength (μ) to the desired value. In some series of mixtures, the sodium chloride was added before the acid and alkali. All additions of solutions were made with "Agla" micrometer syringes. The final mixtures were transferred under hydrogen to the electrode vessel when the E.M.F. (E_{DNA}) was measured with a D.C. potentiometer (Muirhead D72A, Weston cells as standards). Several solutions of the same composition were independently prepared with a reproducibility in E_{DNA} of ± 1 mv or better, depending on the pH of the mixture. For each solution of nucleate investigated the E.M.F. (E_b) of a blank solution, of the same composition but with the nucleate omitted, was also measured. The silver–silver chloride electrodes fatigued rapidly in alkaline solutions of nucleate but reproducible results for ($E_b - E_{DNA}$) were obtained by first measuring E_{DNA} and then using the same electrodes to determine the corresponding E_b.

The cells containing nucleate solution may be written as

$$(-)\ H_2(Pt)|DNA,\ NaCl,\ and\ NaOH\ or\ HCl|AgCl/Ag\ (+),$$

and E_{DNA} and E_b are related by:

$$(E_{DNA} - E^\circ_{DNA}) - (E_b - E^\circ_b) = \frac{2{\cdot}303\boldsymbol{RT}}{\boldsymbol{F}} \cdot \log \frac{[H^+]'[Cl^-]'\gamma'_{H+}\gamma'_{Cl-}}{[H^+][Cl^-]\gamma_{H+}\gamma_{Cl-}} \quad . \quad . \quad (5)$$

where E°_{DNA} and E°_b are the standard potentials (determined from separate measurements in hydrochloric acid) of the particular electrodes used to determine E_{DNA} and E_b, respectively; square brackets and γ denote molar concentrations and molar ionic activity coefficients, respectively; primed quantities refer to the blank solution. The activity coefficients in equation (5) cancel since the ionic strengths in the nucleate and in the blank solutions are virtually identical apart from a small contribution ($\sim$0·005) from the nucleate ions. The proportion of chloride ions bound by the nucleate ion is negligible [18] and so the chloride concentration is the same in both solutions. It then follows from (5) that:

$$(E_{DNA} - E^\circ_{DNA}) - (E_b - E^\circ_b) = \frac{2{\cdot}303\boldsymbol{RT}}{\boldsymbol{F}} \cdot \frac{[H^+]'}{[H^+]} = \frac{2{\cdot}303\boldsymbol{RT}}{\boldsymbol{F}} \log \frac{[OH^-]}{[OH^-]'} \quad . \quad (6)$$

Since only the free $[H^+]$ or $[OH^-]$ is unknown, these may be evaluated and then give the number of equivalents of acid or alkali bound by a standard amount of nucleate containing 4 g.-atoms of phosphorus.

In univalent metal chloride solutions $\gamma_{M+} = \gamma_{Cl-} = \gamma^{\frac{1}{2}}_{MCl}$, where γ_{M+}, γ_{Cl-} are the appropriate activity coefficients.[41] In order to define a pH scale for the present work, this relation is assumed to apply to hydrochloric acid solutions, *i.e.*, $\gamma_{H+} = \gamma_{Cl-} = \gamma_{\pm,HCl}$ and a pH scale is defined by $pH_a = -\log [H^+]\gamma_{\pm,HCl}$, where $\gamma_{\pm,HCl}$ is the mean ionic activity coefficient for hydrogen and chloride ions in a nucleate solution. Since the ionic strength is the same, this mean activity coefficient must have the same value as in the corresponding blank solution (*i.e.*, $\gamma_{\pm,HCl} = \gamma'_{\pm,HCl}$) and may be obtained from

$$E_b = E^\circ_b - \frac{2{\cdot}303\boldsymbol{RT}}{\boldsymbol{F}} \log [H^+]'[Cl^-]'(\gamma'_{\pm,HCl})^2$$

For alkaline solutions the pH on this scale (pH_a) is determined from the free $[OH^-]$, from the value of the activity product of water at the appropriate ionic strength [42a] and on the assumption that $\gamma_{H+} = \gamma_{OH-} = \gamma_{\pm,HCl}$ in a solution consisting chiefly of sodium chloride. In the figures showing titration curves obtained by this method the size of the circles is considerably greater than the errors involved and each point is the average of several determinations. Below pH 4·5 and above pH 10·5, the variation in the determined pH_a values for independent preparations of a given mixture was less than 0·01 unit, and at 4·5—5·0 and 10·0—10·5 the variation was 0·03 unit. The variation in the determined number of equivalents of acid or alkali bound (per 4 P g.-atoms) was never more than 0·03 unit.

Continuous Titrations in Cells with a Liquid Junction.—These were carried out at 25° with solutions of nucleate (0·141 mg. of P/ml.) in sodium chloride (0·05M). General procedures were

[41] Guggenheim, *J. Amer. Chem. Soc.*, 1932, **54**, 1350.

[42] Harned and Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold Publ. Corp., New York, 1943, pp. (*a*) 485, (*b*) 320.

as described previously [5] with the additional precautions mentioned in the Discussion. The pH scale used was the " saturated calomel scale " of Hitchcock and Taylor.[42b] Titrations were considered unsatisfactory unless, when finished, the electrode system recorded the pH's of the buffers to within 0·02 unit of their assigned values. The " water corrections " [43] for the titration curves were obtained by blank titrations of 0·05M-sodium chloride with the same acid and alkali. Under these conditions of controlled ionic strength there was no correction factor for activity coefficients. In the present apparatus the maximum increase in salt concentration due to diffusion from the salt bridge during the 2—3-hour titrations was never greater than 0·01M, which at $\mu = 0{\cdot}05$ would affect the pH by only 0·02 unit at most. The standard deviation computed for 12 independent titrations (curves I, II, Fig. 7) was 0·02—0·04 equiv./4P g.-atoms for pH 3—11, but exceeded 0·05 for pH's outside this range.

Alkaline Hydrolysis.—Nucleate solutions (final concn. = 0·141 mg. of P/ml.; final $\mu = 0{\cdot}02$) were maintained at 25° and pH 12 in an atmosphere of hydrogen for various periods. The containing vessel was made of platinum since Pyrex glass was attacked by alkali. To minimise evaporation the purified hydrogen stream was passed first through the corresponding blank solution, then over the nucleate solution, and finally through a water trap. After this treatment with alkali, amounts of acid were added to adjust the pH to values of about 4, 5, or 10 where the nucleate was known to be stable. The E.M.F.'s were then measured. The E.M.F. of blank solutions treated in the same way was found to be constant even for solutions kept at pH 12 for 24 hr., provided a platinum vessel was used.

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Edgbaston, Birmingham, 15. [*Received, November 3rd*, 1955.]

[43] Jordan and Taylor, *J.*, 1946, 994.