742. *Electrometric Titration of the Sodium Salts of Deoxyribonucleic Acids. Part I.*

By W. A. Lee and A. R. Peacocke.

The sodium salts of the deoxypentose nucleic acids of calf thymus, herring sperm, wheat germ, and mouse sarcoma have been titrated electrometrically at 25° and the effects of dialysis and of drying have been examined. The forward-titration curves of the original nucleic acid solutions (pH 6—7) differ markedly from the back-titration curves after acid and alkali treatment. The back-titration curves from pH 2 and from pH 12 differ significantly from each other and this can be explained in terms of hydrogen bonding or, more probably, by the existence of a slow hydrolysis of some internucleotide linkages at alkaline pH.

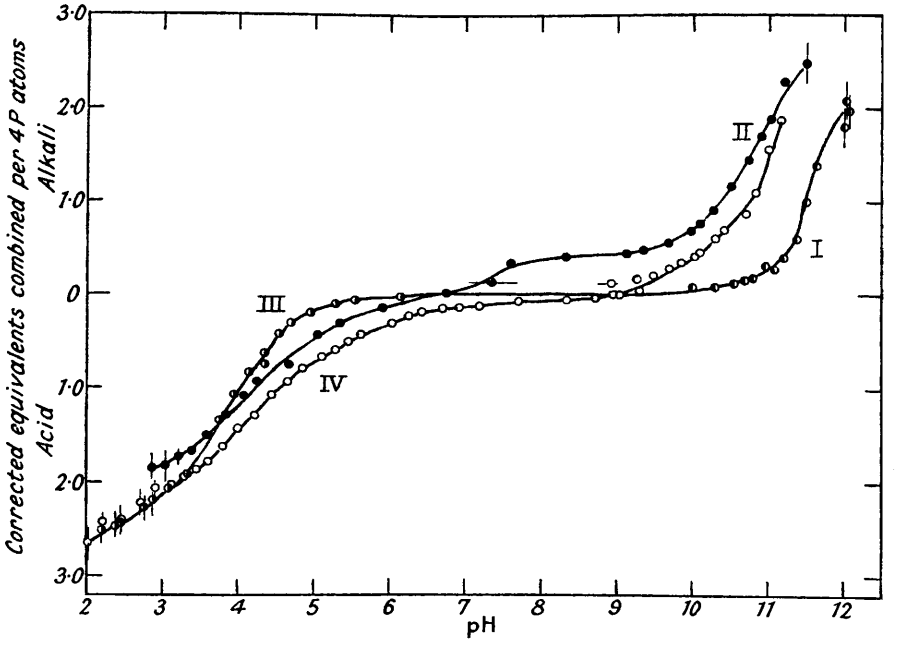
By combining analyses and titration data, an estimate of the proportions of the various types of phosphoryl linkages has been made and this implies a higher frequency of branching of the polynucleotide chain, at triply bonded phosphorus atoms, than has previously been assumed. The effect of acid and alkali on various labile linkages and the dissociation of these substances as polyelectrolytes are discussed.

In recent years techniques have been developed which have made it possible to isolate deoxyribonucleic acids from biological sources with the minimum of degradation. These methods are mainly variations of the procedure of Mirsky and Pollister (*Proc. Nat. Acad. Sci.*, 1942, **28**, 344) whereby deoxyribonucleoprotein can be isolated under neutral conditions from a variety of tissues by utilising the dependence of its solubility in water on the concentration of sodium chloride. The protein may then be separated from the nucleic acid by removing it as a gel with chloroform (Sevag, Lackmann, and Smolens, *J. Biol. Chem.*, 1938, **124**, 425) or by precipitating it with saturated sodium chloride (Hammarsten, *Biochem. Z.*, 1924, **144**, 383). Both methods finally yield white fibrous deoxyribonucleic acid, which dissolves to give highly viscous, non-Newtonian solutions exhibiting negative streaming birefringence (Gulland, Jordan, and Threlfall, *J.*, 1947, 1129; Jordan, 27th Ann. Rep. British Empire Cancer Campaign, 1949, 26; Signer and Schwander, *Helv. Chim. Acta*, 1949, **32**, 853).

It has been observed (Gulland, Jordan, and Taylor, *J.*, 1947, 1131; Cosgrove and Jordan, *J.*, 1949, 1413; Signer and Schwander, *loc. cit.*) that the curves obtained when neutral solutions of the sodium salts of deoxyribonucleic acids, isolated by such mild methods, are first titrated to pH 2·5 and pH 12 differ markedly from those obtained on back-titration with alkali and acid, respectively. Gulland, Jordan, and Taylor (*loc. cit.*) attributed this difference to the existence of hydrogen bonds between titratable groups and, on this hypothesis, titration curves afford a useful method for examining the state of hydrogen bonding of deoxyribonucleic acids.

FIG. 1.

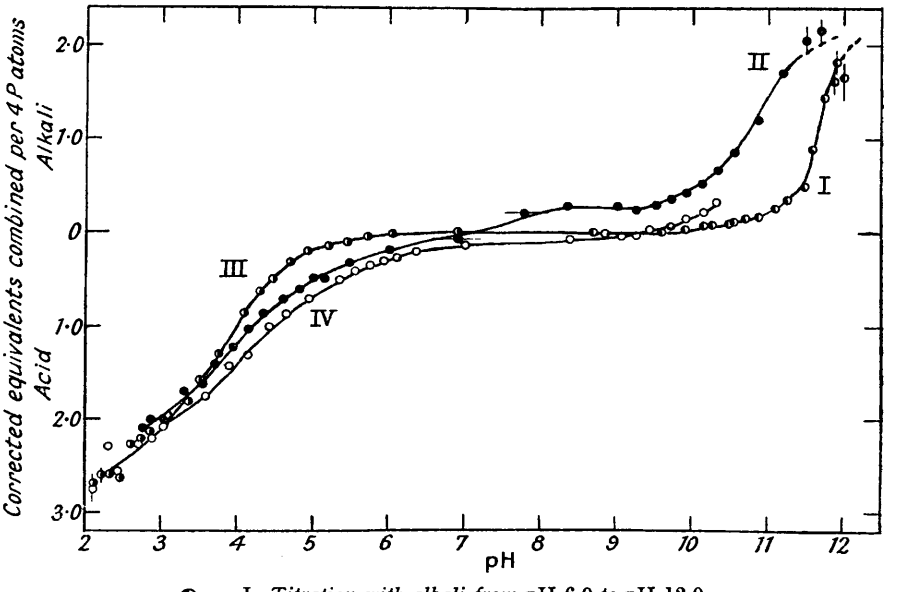
The titration curve of the sodium salt of deoxyribonucleic acid from calf thymus (T_2)*—undried.*



◐ I. *Titration with alkali from* pH 7·2 *to* pH 12·1.
● II. *Back-titration with acid from* pH 12·1.
◑ III. *Titration with acid from* pH 6·8 *to* pH 2·0.
⊙ IV. *Back-titration with alkali from* pH 2·0.

FIG. 2.

The titration curve of the sodium salt of deoxyribonucleic acid from herring sperm (H_1)*—undried.*



◐ I. *Titration with alkali from* pH 6·9 *to* pH 12·0.
● II. *Back-titration with acid from* pH 12·0.
◑ III. *Titration with acid from* pH 6·9 *to* pH 2·0.
⊙ IV. *Back-titration with alkali from* pH 2·0.

The present work on titration curves forms part of an investigation of deoxyribonucleic acids isolated from various sources by mild neutral methods. The effects on their structures of procedures used in isolating nucleic acids have also been examined. Dialysis has little effect, drying a more noticeable one, while ultrasonic irradiation causes a breakdown of the hydrogen bonding, according to titration and other criteria. The results of the irradiation experiments are reported in the succeeding paper (cf. Overend and Peacocke, *Trans. Faraday Soc.*, 1950, **46**, 794). The titrations of unirradiated deoxyribonucleic acids confirm Overend and Peacocke's observations that the forward- and the back-titration curves differ greatly from each other. However, a general feature of all the present results has been the separation of the curves obtained on back-titration from pH 2 and pH 12. The validity of this separation is examined below and possible explanations are discussed.

The quantitative analysis of the constituent purines and pyrimidines of nucleic acids has been placed on a firm basis by the application of the methods of paper chromatography to the separation of nucleic acid hydrolysis products (Vischer and Chargaff, *J. Biol. Chem.*, 1948, **176**, 703, 715; Markham and Smith, *Nature*, 1949, **163**, 250; *idem*, *Biochem. J.*, 1949, **45**, 294; Wyatt, *ibid.*, 1951, **48**, 584). The nucleic acids discussed in this paper have been analysed by these methods (Laland, Overend, and Stacey, in the press) and the results enable a more accurate estimate of the proportion of secondary monoester phosphoryl groups to be made from the titration curves than has been possible hitherto. The values calculated imply a greater degree of branching of deoxyribonucleic acids than has been previously assumed.

Titrations of the Sodium Salts of Deoxyribonucleic Acid.—Figs. 1—4 show the titration curves of the sodium salts of deoxyribonucleic acids isolated from calf thymus glands (T_2), herring sperm (H_1), and wheat germ, and of a sample of the sodium salt of calf thymus nucleic acid kindly supplied by Professor Signer of Berne University (for details of the preparation, see Signer and Schwander, *loc. cit.*). Curves I and III throughout refer to the forward titrations with alkali and acid to pH 12 and pH 2, respectively. Curves II and IV refer to the corresponding back titrations from pH 12 and pH 2. The ordinates in the figures represent the number of equivalents of alkali and acid bound by a standard amount of nucleic acid, containing 4 g.-atoms of phosphorus. Below pH 5 and above pH 9 allowance has been made for the added acid and alkali uncombined with the nucleic acid (the " water correction ") by Jordan and Taylor's method (*J.*, 1946, 994). In the calculations of the ionic strength in the titration solution that this method requires, the simplifying assumption was made that each ionised group in the nucleic acid contributed independently to it. This is justified by the experience of protein titrations (Cohn and Edsall, " Proteins, Amino Acids and Peptides," Reinhold Publ. Co., 1943, p. 453; Cannan, Kibrick, and Palmer, *Ann. N.Y. Acad. Sci.*, 1941, **41**, 247) and, since the ionic strength factor differs appreciably from unity only when the water correction is relatively small, this uncertainty does not affect the accuracy of the results over the pH range 3—11. Outside this range, the water correction is relatively large and gives rise to an increasing uncertainty in the value of the ordinate. The size of the circles and of the vertical lines represents the errors caused both by the uncertainty in the ionic strength of the solution and by the error of the pH measurements.

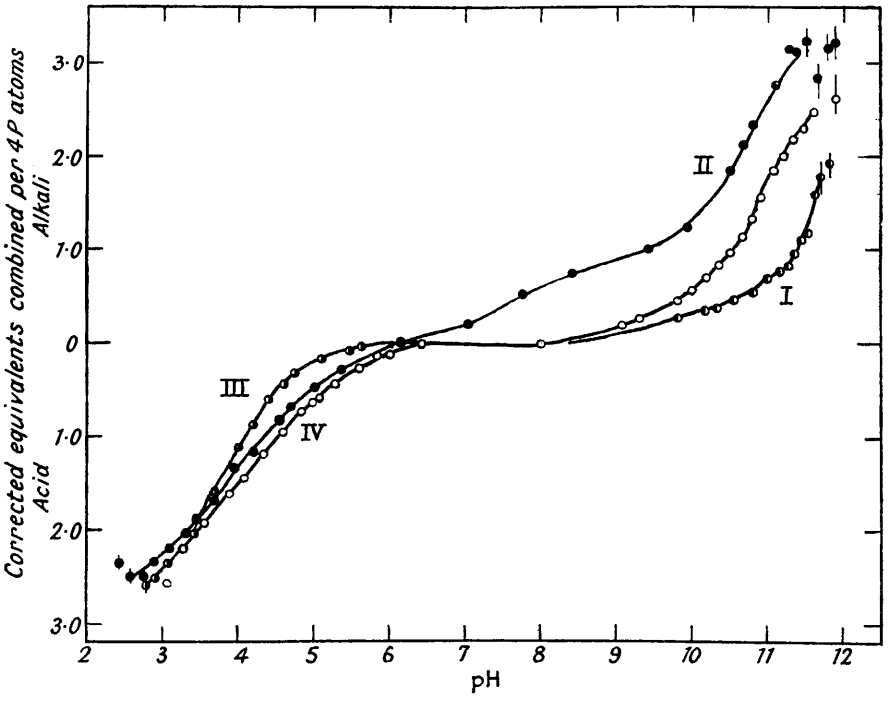
The measured pH values were steady over all the titration range, except in the most alkaline solutions, where there was a tendency for the pH to drop. As carbon dioxide was rigorouslv excluded in all titrations, this observation was confirmed in separate experiments in which sufficient alkali was added to nucleic acid solutions to bring the pH to 12. In the interval between the 3rd minute after adding alkali and 40 minutes later, the pH of the various solutions decreased by 0·07—0·12 unit.

The results confirm the observations by Gulland, Jordan, and Taylor that in the forward titrations there is a rapid liberation of titratable groups above pH 11 (I) and below pH 5 (III) and that, once liberated, these groups are titratable over the whole pH range (II and IV). The number of groups titrated in the acid region is 2·4—2·8 and in the alkaline region is approximately 2. The separation of the back-titration curves II and IV is a noticeable feature of Figs. 1—4 and is outside the range of the errors already discussed. Curve II implies more alkali or less acid combined with the nucleic acids at a given pH than does IV. The results in Fig. 4 agree with the titration curves of this material already published by Signer and Schwander (*loc. cit.*). Unfortunately in the latter work, II and IV (their curves 2 and 4) were not extended far enough for a test of coincidence to be made.

The sodium salt of a deoxyribonucleic acid isolated from mouse sarcoma by the methods of Mirsky and Pollister (*loc. cit.*) and of Sevag, Lackmann, and Smolens (*loc. cit.*) was also titrated. The relative disposition of the titration curves was identical with those of Figs. 1—4, but,

FIG. 3.

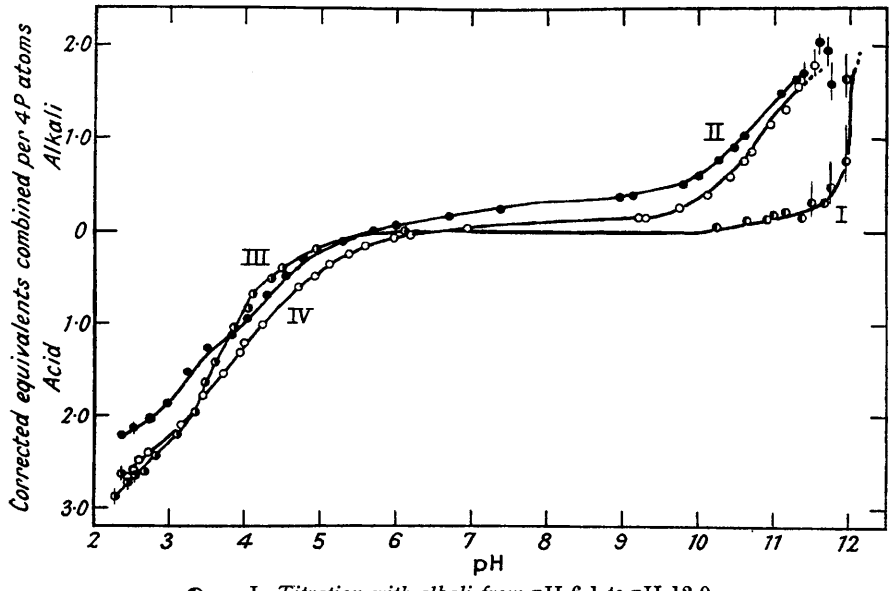
The titration curve of the sodium salt of deoxyribonucleic acid from wheat germ—undried.



◐ I. *Titration with alkali from* pH 6·4 *to* pH 12·0.
● II. *Back-titration with acid from* pH 12·0.
◑ III. *Titration with acid from* pH 6·4 *to* pH 2·4.
⊙ IV. *Back-titration with alkali from* pH 2·4.

FIG. 4.

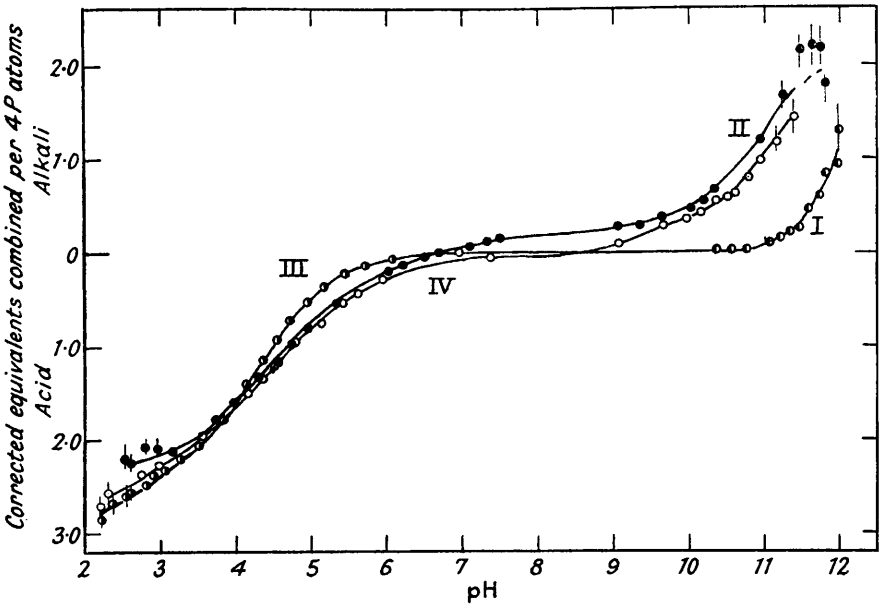
The titration curve of the sodium salt of deoxyribonucleic acid prepared from calf thymus by Signer and Schwander (loc. cit.)*—undried.*



◐ I. *Titration with alkali from* pH 6·1 *to* pH 12·0.
● II. *Back-titration with acid from* pH 12·0.
◑ III. *Titration with acid from* pH 6·1 *to* pH 2·3.
⊙ IV. *Back-titration with alkali from* pH 2·3.

FIG. 5.

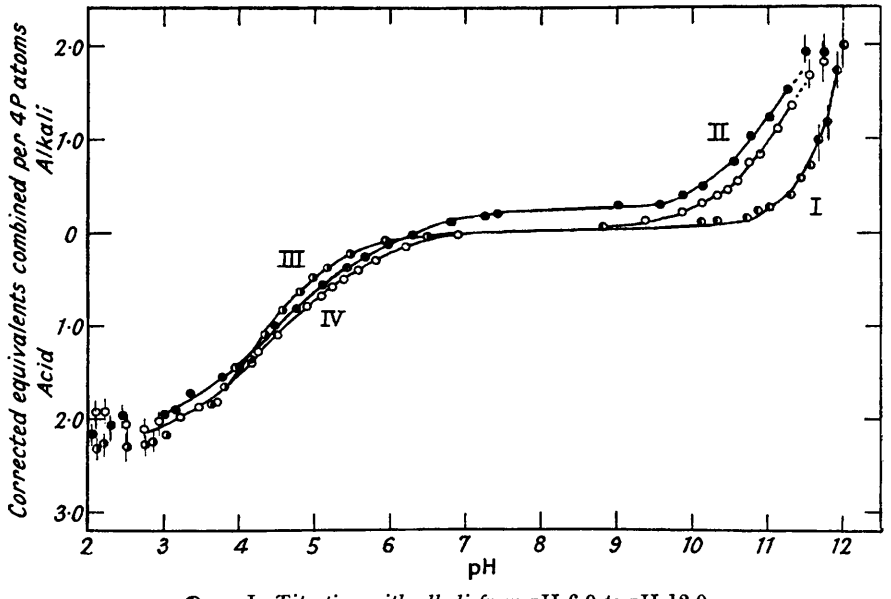
The titration curve of the sodium salt of deoxyribonucleic acid from calf thymus (T_1)*—dried.*



◐ I. *Titration with alkali from* pH 6·9 *to* pH 12·0.
● II. *Back-titration with acid from* pH 12·0.
◑ III. *Titration with acid from* pH 6·9 *to* pH 2·0.
⊙ IV. *Back-titration with alkali from* pH 2·0.

FIG. 6.

The titration curve of the sodium salt of deoxyribonucleic acid from herring sperm (H_1)*—dried.*



◐ I. *Titration with alkali from* pH 6·9 *to* pH 12·0.
● II. *Back-titration with acid from* pH 12·0.
◑ III. *Titration with acid from* pH 6·9 *to* pH 2·0.
⊙ IV. *Back-titration with alkali from* pH 2·0.

owing to the high viscosity of the solutions and foaming at alkaline pH, the results above pH 7 are not yet considered sufficiently reliable. Below this pH, the curves are quantitatively very similar to those of Fig. 1. Herring-sperm nucleic acid was also titrated in the presence of potassium chloride (1·0M.) at alkaline pH (see Discussion).

The Effect of Dialysis.—Dialysis, followed by drying in the frozen state, is frequently employed during extractions of deoxyribonucleic acids in order to remove any contaminating sodium chloride. Calf thymus and herring sperm nucleic acids were therefore titrated before (as T_1 and H_1) and after (as T_2 and H_2) dialysis and drying in the frozen state. There was no alteration in the shape and relative position of curves I—IV or in the number of groups titrated in the various pH ranges. There were only minor quantitative differences between the two sets of results and these are of doubtful significance. (N.B. Fig. 1 refers to a dialysed nucleic acid and Fig. 2 to an undialysed acid.) It was shown in other dialysis experiments in sodium chloride solutions of the nucleic acids that the release of inorganic phosphate during dialysis was less than 1·6% of the total phosphorus present and that there was no significant change in the N : P ratio in the nucleic acid solution. Dialysis therefore has only a negligible effect on those properties of nucleic acids which are detectable by titration.

The Effect of Drying.—In the experiments of Gulland, Jordan, and Taylor and of Cosgrove and Jordan, the nucleic acids were dried over phosphoric oxide (110°, 30 minutes, 0·01 mm. of mercury) before dissolution and titration (*loc. cit.*; Jordan, personal communication). In the experiments reported here, however, undried deoxyribonucleic acids containing some moisture (about 15%) were dissolved for titration and the solutions subsequently analysed for phosphorus. The calf thymus and herring sperm deoxyribonucleic acids which were used in our experiments were therefore also titrated after drying by the same method. The titration curves (Figs. 5 and 6) are very similar quantitatively and qualitatively to those obtained after drying by the method mentioned. In particular, the degree of coincidence of the back titration data with acid and alkali is the same, as shown by an examination of the experimental points of the other investigators. Comparison of Figs. 5 and 6 with Figs. 1 and 2 suggests that after the drying the size of the loop formed by I and II and by III and IV is slightly reduced. On the hypothesis already mentioned, this would imply a small reduction in hydrogen bonding caused by drying. The separation of II and IV appears to be greater if the deoxyribonucleic acids are not previously dried.

Discussion.

(a) *Estimation of the Quantity of Secondary Monoester Phosphoryl Groups.*—The work of Simms (*J. Amer. Chem. Soc.*, 1926, **48**, 1239), von Muralt (*ibid.*, 1930, **52**, 3518), and Wyman (see Cohn and Edsall, *op. cit.*, p. 451) has shown that it is possible to represent the titration curve of a polybasic acid in terms of a series of titration constants, G_1'. These constants are the apparent dissociation constants (involving the activity of the hydrogen ion and the concentrations of other molecular species) of the monobasic acids which, mixed in the appropriate proportions, would have the same titration curve as the polybasic substance under consideration. For reasons given by these authors titration constants can be identified with the dissociation constants of particular chemical types of groups, if there is no marked electrostatic or other interaction between dissociating groups. This assumption has frequently been made (Levene and Simms, *J. Biol. Chem.*, 1926, **70**, 327; Fletcher, Gulland, and Jordan, *J.*, 1944, **33**; Gulland, Jordan, and Taylor, *loc. cit.*) in order to estimate from nucleic acid titration curves the number of secondary phosphoryl groups, an important quantity not obtainable by direct analytical methods. The procedure is not unambiguous because different combinations of titration constants and the corresponding equivalents of monobasic acids give theoretical titration curves equally coincident with that observed. Another uncertainty arises from the interactions between dissociating groups which must occur in such a highly charged molecule as nucleic acid (Jordan, *Trans. Faraday Soc.*, 1950, **46**, 793).

The error in an estimation of the secondary phosphoryl groups due to the former uncertainty can be greatly reduced when independent analytical determinations of all other titratable groups are available. By chromatographic methods, determinations have been made of the individual constituent bases of the titrated nucleic acids (Laland, Overend, and Stacey, *loc. cit.*) and these give the number of titratable amino-groups, per 4 g.-atoms of nucleic-acid-phosphorus, contributed by guanine, adenine, cytosine, and 5-methyl cytosine. The procedure was then as follows. The titration constants of these amino-groups were taken, as a first approximation, to be similar to the dissociation constants of the same group in the corresponding ribonucleotides, *i.e.*, pG' = 2·3, 3·7, and 4·2, respectively (Levene and Simms, *J. Biol. Chem.*, 1925, **65**, 519), since no data on the deoxyribonucleotides are available. The number of

TABLE I.

The titratable groups of the sodium salts of deoxyribonucleic acids.

Source of nucleic acid and treatment before titration :		Calf thymus (undried)		Calf thymus (dried)		Herring sperm (undried)		Herring sperm (dried)		Wheat germ (undried)
Figure :		1	1	5	5	2	2	6	6	3
Curve :		II	IV	II	IV	II	IV	II	IV	IV §
Groups										
1. Amino. Guanine	pG'_1	2·5	2·5	2·5	2·5	2·35	2·35	2·6	2·6	2·9
	Equivs./4P †	0·75	0·75	0·75	0·75	0·71	0·71	0·71	0·71	0·66
2. Amino. Adenine	pG'_2	3·7	3·7	3·85	3·85	3·70	3·70	3·85	3·85	3·85
	Equivs./4P †	1·02	1·02	1·02	1·02	1·0	1·0	1·0	1·0	0·90
3. Amino. Cytosine and 5-methyl cytosine ‡	pG'_3	4·75	4·75	4·85	4·85	4·85	4·85	4·90	4·90	4·5
	Equivs./4P †	0·84	0·84	0·84	0·84	0·92	0·92	0·92	0·92	0·84
4. Secondary phosphoryl groups	pG'_4	7·0	6·5	6·15	6·00	7·00	6·5	6·5	6·5	5·5
	Equivs./4P	0·54	0·33	0·59	0·40	0·28	0·18	0·29	0·27	0·42
5. Enolic hydroxyl groups of guanine and thymine *	Equivs./4P †	1·76	1·76	1·76	1·76	1·76	1·76	1·76	1·76	1·57

* Obtained from analytical data.

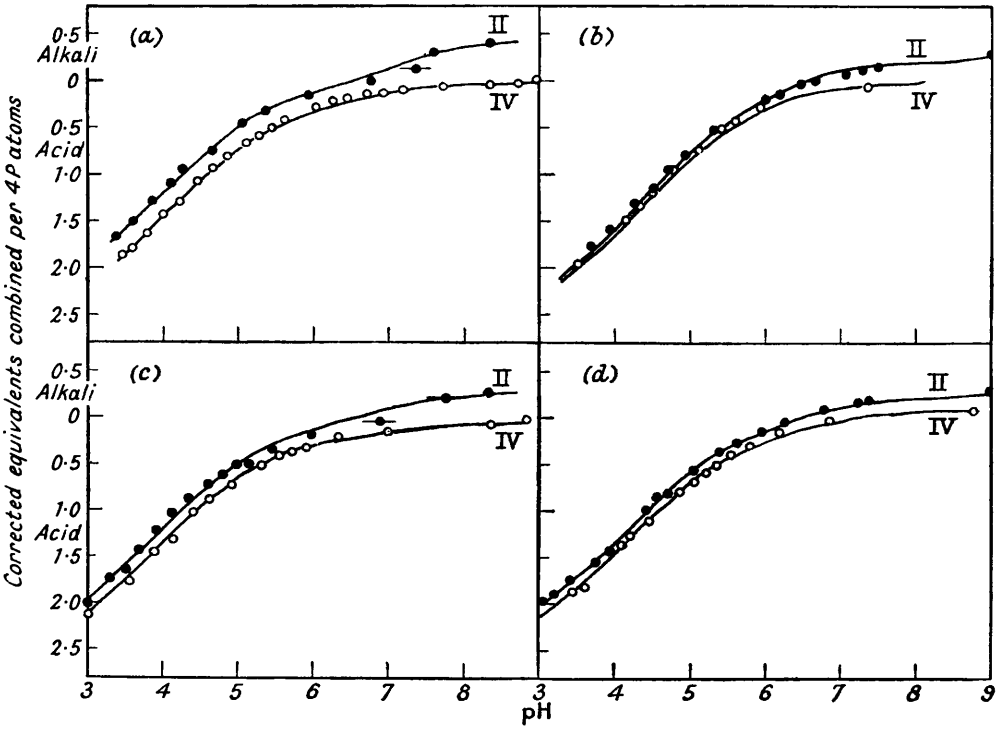
† pG' of amino-group of 5-methylcytosine assumed to be the same as in cytosine.

‡ No pG'_5 given because of the impossibility of representing titration curve above pH 9 by one titration constant only.

§ No detailed analysis was made of curve II for wheat germ nucleic acid, owing to the difficulty of finding a suitable pG'_4 value. The number of secondary phosphoryl groups is high, ~0·6—0·9.

FIG. 7.

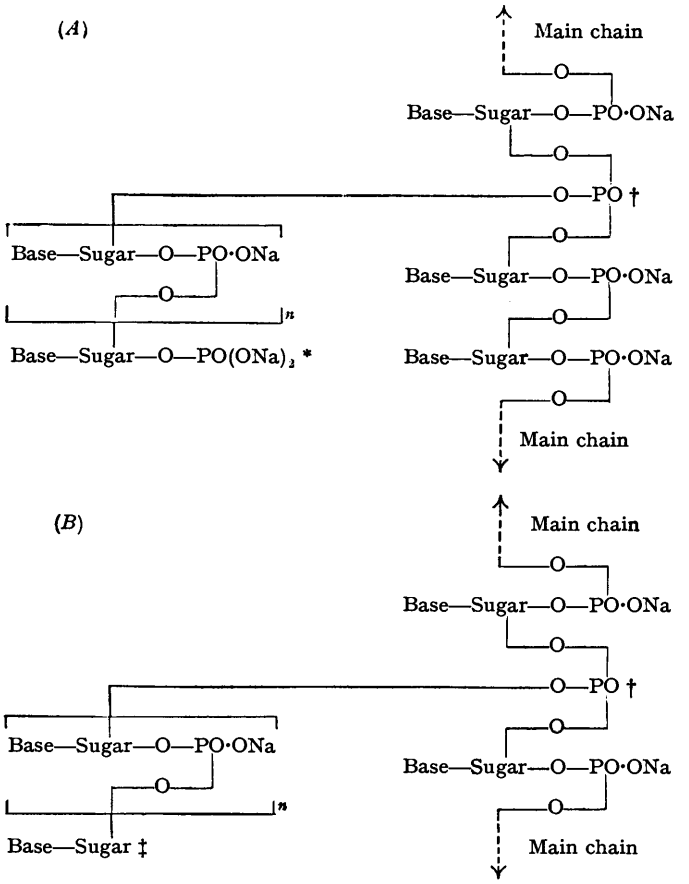
Comparison of calculated titration curves with experimental data. Smooth curves are calculated by using the quantities in Table I and are adjusted in position on the ordinate so as to coincide with the experimental values at the inflexion point (pH 8—9).



Circles are the experimental data from (*a*) Fig. 1; (*b*) Fig. 5; (*c*) Fig. 2; (*d*) Fig. 6.

● II. *Back-titration with acid after alkali treatment.*
⊙ IV. *Back-titration with alkali after acid treatment.*

equivalents of alkali required to titrate such groups from a fixed lower pH, *e.g.*, 3·5, to a fixed higher pH, *e.g.*, 8·5, corresponding to the point of inflexion in the titration, was then calculated by Simms's method (*loc. cit.*) and was invariably less than that experimentally observed. Since no deamination occurs during the titration, this difference must have been due to secondary phosphoryl groups, the only other group dissociating to any appreciable extent in this pH range (pG' = 5·9—7·0; Levene and Simms, *loc. cit.*; Kumler and Eiler, *J. Amer. Chem. Soc.*, **1943**, **65**, 2355). By assuming a value of 6—7 for the pG', the number of secondary phosphoryl groups was estimated and the titration curve below the inflexion point was calculated on this basis. If the calculated and observed curves did not have the same shape and curvature, the assumed titration constants were modified and this process of successive approximations was continued until a curve parallel with that observed was obtained. The amount of secondary phosphoryl groups assumed in this last calculation was then the best estimate that could be made from a given experimental curve and had an overall probable error of 0·07 per 4 phosphorus atoms. Alterations in the values taken for the various titration constants were limited by the necessity of obtaining a curve coincident with that observed. The results of the calculations are summarised in Table I, and Fig. 7 shows the theoretical curves and experimental data in the more important cases. It is clear from Fig. 7 that the curves below the inflexion point can be accurately represented as the titration of amino- and secondary phosphoryl groups. The analytically determined numbers of enolic hydroxyl groups (Table I) is in only approximate agreement with the maximum equivalents of base bound (1 and 2), owing to the difficulty of making accurate calculations of the water correction at the highest pH and probably to the presence of a slow reaction with alkali (see below).



* Secondary phosphoryl group. ‡ Sugar with one free hydroxyl group.
† Triesterified phosphoric acid residue — branching point.
Base = adenine, guanine, cytosine, 5-methyl cytosine, or thymine.

Gulland, Jordan, and Taylor (*loc. cit.*), and Cosgrove and Jordan (*loc. cit.*), give 0·25 as the maximum number of secondary phosphoryl groups for every four atoms of phosphorus in calf-thymus and herring-sperm nucleic acids (dried) but considered the actual number to be probably less than this. The number of secondary phosphoryl groups given in Table I are, in general, at least as great as this maximum value and the difference chiefly arises from the assumption by these workers that there were 3·0 amino-groups for every four atoms of phosphorus, whereas the analytical results now show that there are less than this amount, namely 2·61 and 2·63 in the above two nucleic acids [compare the values of 2·60 and 2·64, respectively, found by Wyatt (*loc. cit.*) with these nucleic acids].

(b) *Structure.*—The number of secondary phosphoryl groups calculated from titration curves II and IV can only represent the number of these groups in the units present after alkali and acid treatment, when labile macromolecular linkages which are susceptible to these agents have already been ruptured (see below). However, it is still of interest to enquire into the mode of linking of nucleotides in these units, and, indeed, most discussions of the past have been primarily concerned with them. Since alkali, even under the mild titration conditions, may break some internucleotide linkages (see below), only the numbers of secondary phosphoryl groups deduced from curves IV will be employed in the following discussion. Table I shows that about one in every 10—20 phosphorus atoms possesses a secondary phosphoryl group. These groups cannot be situated solely at the ends of unbranched chains since a degree of polymerisation of only 10—20 nucleotides would not account for the high molecular weights (*e.g.*, $8·2 \times 10^5$, Cecil and Ogston, *J.*, 1948, 1382) observed with deoxyribonucleic acids prepared in this way. Branching of the main polynucleotide chain must consequently be occurring, with each branch terminated by a singly esterified phosphoric acid residue. Since the 2-deoxy-D-ribose moiety, unlike the D-ribose in ribonucleic acids, has only two groups (3- and 5-) available for internucleotide linkages (Lythgoe and Brown, *J.*, 1950, 1990), this branching must arise at triply bound phosphorus atoms, as in formula (*A*), which has been written on the assumption that there are no free sugar hydroxyl groups.

The frequency with which branching of the main chain occurs will depend on the number (n) of nucleotides in the side chains and cannot be determined by this type of end-group analysis alone. There is ample evidence that the nucleic acid molecule is extremely asymmetric (*e.g.*, Signer, Caspersson, and Hammarsten, *Nature*, 1938, **141**, 122) and of high molecular weight, so only a negligble proportion of the secondary phosphoryl groups will be those at the end of the long main polynucleotide chain.

The proportions of phosphoric acid residues esterified to different degrees may be calculated from the data of Table I and the ratio of sodium to phosphorus atoms. Of every four phosphorus atoms, let a = the number present as $RO{\cdot}PO(ONa)_2$, the number of secondary phosphoryl groups given in Table I (curves IV); b = the number present as $(RO)_2PO{\cdot}ONa$; and c = number present as $(RO)_3PO$, where R = a nucleoside residue. By definition, $(a + b + c) = 4$. Also, since the nucleic acid is isolated at pH 6—7 in the presence of sodium chloride only, all the primary and secondary phosphoryl groups will be present in their charged form (see curves III), combined with sodium. So, $b = (d - 2a)$, where d is the number of sodium atoms for every 4 phosphorus atoms, by analysis. The two equations enable b and c to be calculated (Table II).

TABLE II.

Proportions of different phosphoryl linkages in the sodium salts of deoxyribonucleic acids.

Source of deoxyribonucleic acid and treatment before titration :		Calf thymus (undried)	Calf thymus (dried)	Herring sperm (undried)	Herring sperm (dried)	Wheat germ (undried)
$RO{\cdot}PO(ONa)_2$	a =	0·33 ± 0·07	0·40 ± 0·07	0·18 ± 0·07	0·27 ± 0·07	0·42 ± 0·07
$(RO)_2PO{\cdot}ONa$	b =	3·34 ± 0·16	3·20 ± 0·16	2·84 ± 0·16	2·66 ± 0·16	3·16 ± 0·21
$(RO)_3PO$	c =	0·33 ± 0·17	0·40 ± 0·17	0·98 ± 0·17	1·07 ± 0·17	0·42 ± 0·22
P atoms without corresponding base molecules (by analysis) *		0·38 ± 0·07	0·38 ± 0·07	0·32 ± 0·07	0·32 ± 0·07	0·69 ± 0·07

All figures are calculated for 4 phosphorus atoms.
The probable errors of b and c are high because of the cumulative effect of the calculations.
* Cf. Wyatt, *loc. cit.*

In calf-thymus and wheat-germ deoxyribonucleic acid, a and c are equal and, within the error limits of the method, are also approximately equal to the number of phosphorus atoms without corresponding molecules of base. This is in accordance with (*A*) as the only mode of

branching of the polynucleotide chain. However, in herring sperm nucleic acid, *a* is approximately equal, within the limits of error, to the number of phosphorus atoms with no corresponding molecules of base but both of these figures appear to be significantly less than *c*, because the sodium : phosphorus ratio is less than unity. This implies that a significant number of the triply bound phosphorus atoms in this nucleic acid are linked indirectly to a base molecule but not to a secondary phosphoryl group. This could arise from the occurrence of structures such as (*B*), as well as (*A*), in this sample of herring sperm nucleic acid. In (*B*) there is a free sugar hydroxyl group and, in accordance with this and in contrast with thymus nucleic acid (Gulland, Jordan, and Taylor, *loc. cit.*, Fig. 2, curve III), the titration curve of this herring sperm nucleic acid at pH 12 in 1·0M-potassium chloride (to reduce water-correction errors) showed no inflexion point, which would have appeared if free sugar hydroxyl groups were not being titrated.

(c) *The Back-titration Curves.*—The separation of the back titration curves II and IV has been observed by us in altogether 14 independent titrations of the deoxyribonucleic acids from the sources mentioned. This feature of the titration curve is less noticeable if the nucleic acids are dried before titration, and the view of Gulland, Jordan, and Taylor (*loc. cit.*) that these curves are virtually coincident may be due to a difference in technique of this type. Three types of explanation of the difference can be suggested.

(i) The investigations of Cannan, Kibrick, and Palmer (*loc. cit.*; *J. Biol. Chem.*, 1942, **142**, 803) and of Cohn, Green, and Blanchard (*J. Amer. Chem. Soc.*, 1937, **59**, 509) have shown that increasing the salt concentration causes the titration curves of proteins to rotate around their isoelectric points. Solutions alkaline to this point, the case corresponding to nucleic acid titrations above pH 2·0—2·5, become more acid on the addition of salt and the effect is greater the more negative the charge. Since no attempt has been made to maintain the ionic strength constant during the titrations the possibility arises that variations in ionic strength may have been sufficient in magnitude and sign to cause the difference observed between II and IV.

The calculation of the total ionic strength of a titration mixture at each pH has already been discussed; it is never found to vary by more than 0·005 unit over the full course of the titrations. When the titration is continued for any length of time there is a danger of diffusion of potassium chloride from the end of the saturated salt bridge. This would cause the ionic strength at the end of curves II and IV to be greater than at the beginning by an amount not exceeding 0·03 unit, as determined by experiment. Detailed examination shows that if one allows for this effect, back-titration curves from pH 12 and 2·5 are obtained to replace II and IV which are, if anything, separated more than before, so that other explanations must be sought.

(ii) Gulland, Jordan, and Taylor suggested that the hydrogen bonds in these nucleic acids were ruptured with equal facility by acid and alkali and that they linked two groups, one of which titrated in the acid region and the other in the alkaline region, *e.g.*, the amino-groups and the titratable $-NH-C=O \rightleftharpoons -N=C-OH$ systems of guanine and thymine, respectively. The presence of such bonds can still be regarded as a satisfactory explanation of the major part of the difference between the forward- and the back-titration curves presented here. If, in addition, there were also present smaller numbers of hydrogen bonds which could be broken only by acid or by alkali, then the two back-titration curves would not be coincident: each would represent the titration of nucleic acid molecules with different residual hydrogen bonding. Such hydrogen bonds would have to link groups titrated in the acid range or in the alkaline range to other groups not dissociating during the titration (*e.g.*, primary phosphoryl groups). Some evidence for the existence of these two types of hydrogen bond in deoxyribonucleic acids is available.

After ultrasonic irradiation of herring-sperm nucleic acid the difference between III and IV disappears but continued irradiation, long after the viscosity has ceased to fall, never entirely eliminates the difference between I and II and between I and IV (unpublished work). It appears that hydrogen bonds are present which are broken only by alkali treatment but do not affect titration at acid pH values. Similar curves have been obtained by Gulland, Jordan, and Taylor (*loc. cit.*, Figs. 6, 4, and 5) with certain samples of thymus nucleic acid. More recently, Cavalieri and Angelos (*J. Amer. Chem. Soc.*, 1950, **72**, 4686) have shown that treatment of thymus nucleic acid with acid or alkali (pH 3 and 12) greatly increased the number of primary and secondary phosphoryl groups available for the binding of the cationic dye, rosaniline. These authors therefore postulate that the phosphoryl groups are linked by hydrogen bonds to amino-groups and, we would add, to enolic hydroxyl groups also. It is of interest that alkali and acid treatment were not equally effective in releasing primary phosphoryl

anions and this would support the postulate of two types of hydrogen bond involving primary phosphoryl groups.

This explanation of the separation of II and IV therefore seems to have a reasonable basis and was that previously put forward (Overend and Peacocke, *Trans. Faraday Soc.*, 1950, **46**, 783). However, it has subsequently been shown (following paper) that, although ultrasonic irradiation breaks down almost all of the hydrogen bonding of herring sperm nucleic acid, the separation of II and IV is as great as in the unirradiated sample. Also on this hypothesis and contrary to the evidence of Figs. 1—6, II and IV should become coincident over the pH ranges (about 8) where no groups are being titrated. This implies that some factor other than hydrogen bonding may be chiefly responsible for the separation of II and IV.

(iii) If, in addition to rupture of the hydrogen bonds responsible for the distinction between forward- and back-titration curves, there was also an irreversible neutralisation of acid and alkali, then II would be displaced upwards and IV downwards, causing a separation of the kind observed. Preliminary evidence for such a reaction with alkali is provided by the observed tendency for the pH to decrease slightly in very alkaline solutions; with acid, however, the pH values were quite stable, even in the most acid solutions. No ammonia was detected in alkaline nucleic acid solutions under the titration conditions, so no hydrolysis of amino-groups can have occurred.

Euler and Fonó (*Arkiv Kemi, Min., Geol.*, 1948, **25**, No. 3) have reported that treatment of thymus nucleic acid with alkali at pH 11·4 and 25° caused a release of secondary phosphoryl groups, amounting to 0·9 per 4 phosphorus atoms in 330 minutes. They suggested that the alkali ruptures ester linkages between secondary phosphoryl and enolic hydroxyl groups, but no titration evidence for release of the latter groups was presented. A kinetic analysis by ourselves of their plot of secondary phosphoryl groups formed against hydrolysis time shows that the initial release of groups was at first very rapid (0·36 group/4P at a first-order rate constant of $\sim$1 min.$^{-1}$). This was followed by a slower release of 0·27 group at a first-order rate constant of $5{\cdot}1 \times 10^{-2}$ min.$^{-1}$, and a further 0·27 group at $3{\cdot}0 \times 10^{-3}$ min.$^{-1}$. The initial fast reaction may consist of the breakdown of both hydrogen bonds involving secondary phosphoryl groups, thereby bringing their p*G′* into Euler and Fonó's titration range (pH 5—8), and of labile $C_{(1)}$-phosphate linkages, as discussed below. In the slower reactions with alkali some of the internucleotide linkages must be ruptured in order to form such a high proportion of secondary phosphoryl groups.

The occurrence, under the very similar conditions (pH 12, 25°, 10—20 minutes) of our titration experiments, of similar reactions with alkali would explain both the separation of II and IV and the greater quantity (about 0·1—0·2 by Table I) of secondary phosphoryl groups apparently present after alkali treatment as compared with acid treatment. The separation of II and IV below pH 6 could be caused by the presence, in alkali treated nucleic acid, of extra acidic groups not titrated at pH 2—12, such as primary phosphoryl groups. These could only arise from the hydrolysis by alkali of one of the linkages of triesterified phosphoric acid residues (see *A* and *B*). In this explanation of the curves, the dried nucleic acids must be assumed to be rather more stable towards these various effects of alkali. The sedimentation-constant measurements by Cecil and Ogston (*loc. cit.*) on thymus nucleic acid further support this view of the effect of acid and alkali. Acid treatment (pH 3·5, room temperature) caused some disaggregation and these changes proceeded to a limit, apparently after only 10 minutes' treatment. Alkali treatment (pH 12·5, room temperature), however, caused a progressive and more profound disaggregation, ultimately producing particles too small to sediment. Cavalieri and Angelos (*loc. cit.*, footnote, p. 4690) also noted variations in the behaviour of thymus nucleic acid with different times of exposure to alkali.

It is not possible to decide between explanations (ii) and (iii) from the titration curves alone, by observing if II and IV coincide with each other at the extremes of pH or continue parallel, since the errors are too great in these regions of the curves. Both factors may be involved in the separation of II and IV, although the available evidence suggests that the occurrence of an irreversible reaction is chiefly responsible.

(d) *Labile Linkages.*—The suggestion has been made made (Stacey, Li, and Overend, *Nature*, 1949, **163**, 538; Overend, Stacey, and Webb, forthcoming publication) that a small proportion of the $C_{(1)}$ atoms of 2-deoxy-D-ribose in nucleic acids may be involved in highly labile, polymeric linkages, possibly with phosphoric acid residues, as well as in glycofuranosidic linkages to the bases. Even at 25°, such a linkage would be very labile to acids (Friedkin, *J. Biol. Chem.*, 1950, **184**, 449) and to alkali (Cori, Colowick, and Cori, *J. Biol. Chem.*, 1937, **121**, 465; Farrar, *J.*, 1949, 3131; Foster, Overend, and Stacey, *J.*, 1951, in the press). Complete and rapid

hydrolysis of these bonds should therefore occur with acid and alkali, releasing free phosphoryl groups and the $>C_{(1)}\cdot OH$ group of the sugar, which would be in equilibrium with detectable quantities of the straight-chain *aldehydo*-form (Overend, *J.*, 1950, 2769). Evidence will be presented elsewhere that acid and alkali under the titration conditions do in fact release aldehyde groups by very fast reactions. These reactions, like the rupture of hydrogen bonds between amino- and enolic hydroxyl groups, would cause no difference between II and IV.

The *N*-glycosidic bond between 2-deoxy-D-ribose and the purine bases is known to be unstable in acid solution but it has recently been shown (Volkin, Khym, and Cohn, *J. Amer. Chem. Soc.*, 1951, **73**, 1533) that the deoxyribose analogue of adenylic acid is converted into adenine at the rate of only 2% per hour in 0·01N-hydrochloric acid at room temperature. Hence only negligible hydrolysis of this bond would be expected to occur during the titrations at low pH.

(e) *The Dissociation of Deoxyribonucleic Acids.*—The dissociation of long-chain polybasic acids has attracted increasing attention in recent years (Overbeek, *Bull. Soc. chim. Belg.*, 1948, **57**, 252; Katchalsky, Künzle, and Kuhn, *J. Polymer Sci.*, 1950, **5**, 283; Wall and de Butts, *J. Chem. Physics*, 1949, **17**, 1330) and experimental data on synthetic charged polymers of this type are accumulating (Katchalsky and Spitnik, *J. Polymer Sci.*, 1947, **2**, 432; Arnold and Overbeek, *Rec. Trav. chim.*, 1950, **69**, 192). Qualitatively, one of the main results of this work is that the apparent dissociation constant (pK') of a group is strongly dependent on the concentration and charge of the polybasic acid and on the ionic strength of the solution. The theoretical work of Overbeek (*loc. cit.*, Fig. 1) and the experiments of Arnold and Overbeek (*loc. cit.*, Fig. 1) show that, when the change in shape with varying charge is also taken into account, the pK' of an acidic group may increase steadily with molecular charge (*i.e.*, decreasing acidic strength), or increase at first and then slowly decrease again, according to the ionic strength.

The pK' values (= pG', because no other groups are being titrated in this region) of the enolic hydroxyl groups in the present nucleic acid titrations above pH 9, when the negative charge on the molecule is relatively large, show an initial increase and then decrease slightly again (cf. Jordan, *loc. cit.*, 1950). The decrease, however, occurs in the pH region above 11 where the experimental data are least precise. An exact comparison of these results with published theoretical predictions is not possible but they indicate that titrations of nucleic acids under conditions of controlled ionic strength should yield useful information about the degree to which nucleic acids may be represented by various theoretical molecular models. Because of this variation of dissociation constant, and hence of titration constant, with pH too much weight must not be attached to the titration constants in Table I; they can only represent average values for each group over certain pH ranges.

It is hoped to isolate sufficiently large quantities of deoxyribonucleic acids to extend this work to titrations at controlled ionic strengths in order to provide data for comparisons with theory and for a more quantitative study of the importance of the various explanations of the back-titration curves. Eventually such data will also be important in analyses of the effect of ionic strength on viscosity and in studies on the importance of hydrogen bonding in the macromolecular structure of these nucleic acids.

Experimental.

Preparation of the Sodium Salts of the Deoxyribonucleic Acids.—Nucleic acid (T_1) from thymus gland was prepared by a combination of the methods of Mirsky and Pollister (*loc. cit.*) and of Sevag, Lackmann, and Smolens (*loc. cit.*). The nucleic acid was precipitated from saline solutions by the addition of ethanol, washed with 90% ethanol to remove salts, and dried in a desiccator.

Nucleic acid (H_1) from soft herring roe was obtained from a solution of nucleoprotein, isolated by the method of Mirsky and Pollister (*loc. cit.*), by precipitation of the protein with excess of sodium chloride, centrifugation, and precipitation of the nucleic acid by the addition of ethanol. The nucleic acid was washed with 90% ethanol to remove salts and dried in a desiccator.

Full details of the isolations of T_1 and H_1 nucleic acids, which were carried out by Mr. S. G. Laland, will subsequently be published.

A second sample of nucleic acid from thymus gland (T_2), was prepared by dissolving T_1 (178 mg.) in distilled water (18 ml.) and dialysing it against distilled water (285 ml.) at room temperature for 28 hours. The resulting sample was dried in the frozen state, yielding 140 mg. of material.

A second sample of nucleic acid (H_2), from soft herring roe, was obtained by dissolving sample H_1 (500 mg.) in distilled water (50 ml.), and dialysing it for 28 hours against distilled water (600 ml.) at room temperature. The resulting solution was dried in the frozen state.

Nucleic acid from wheat germ was isolated as described by Laland, Overend, and Webb (*Acta Chem. Scand.*, 1950, **4**, 885).

Composition of the Sodium Salts of the Deoxyribonucleic Acids.—Before analysis, the samples were dried over phosphoric oxide at 110°/0·02 mm. The samples were analysed by Mr. S. G. Laland with the exception of the sodium to phosphorus ratio analysis. Full details of the methods used will be published. Results are recorded in the tables.

Determination of Sodium.—The sodium salt of the deoxyribonucleic acid (20 mg.) was digested with concentrated sulphuric acid (0·3 ml.) in a Pyrex micro-Kjeldahl flask until completely charred, and then carbonaceous material was oxidised to yield a clear solution by the addition of concentrated nitric acid (2 drops). Heating was continued until no more nitric acid fumes were evolved and the solutions were transferred to platinum crucibles. Water and sulphuric acid were removed by heating over a steam-bath and then over a small flame.

The remainder of the analysis was continued according to the methods of Overman and Garrett (*Ind. Eng. Chem.*, 1937, **9**, 72) for the removal of phosphorus and of Barber and Kolthoff (*J. Amer. Chem. Soc.*, 1928, **50**, 1625) for the determination of sodium.

Titration Procedure.—Solutions were prepared by dissolving air-dry samples in boiled distilled water and analysed for phosphorus as described by Jones. Lee. and Peacocke (*J.*, 1951, 623). The titrations were performed in an atmosphere of nitrogen at 25°, the gas serving both to stir the solutions and to keep them free from carbon dioxide. pH measurements were made with a Cambridge pH meter in conjunction with Cambridge glass electrodes (" Alki " electrodes above pH 9) and a saturated calomel electrode. Electrical contact between the latter and the titrated solution was maintained by a saturated potassium chloride salt bridge.

For the purpose of standardising the pH meter, 0·05M-sodium borate and 0·05M-potassium hydrogen phthalate were assumed to have pH 9·18 and 4·008, respectively. This pH scale is the " saturated calomel scale " of Hitchcock and Taylor (*J. Amer. Chem. Soc.*, 1937, **59**, 1812) and is recommended by Harned and Owen (" The Physical Chemistry of Electrolytic Solutions," Reinhold Publ. Corp., 1943, p. 320). The error in the measurement of pH was ±0·01 unit at all pH values.

	N, %	P, %	N/P, %	Na/P (atoms)	Sodium ribonucleate, %	$\lambda_{max.}$ (mμ) †	η (rel.) ‡
T_1 and T_2	12·8	7·90	1·62	1·0*	1·3	259	3·6
H_1 and H_2	13·40	8·35	1·61	0·80 ± 0·02	0·4	259	1·85
Wheat germ sample	13·70	8·65	1·59	1·00 ± 0·04	4·80	259	1·33

* One estimation only (Gulland, Jordan, and Taylor give the same figure; ratio in Signer and Schwander's thymus nucleic acid, found by us, is 0·98).

† Wave-length of maximum ultra-violet absorption.

‡ Viscosity relative to water measured at 25° in an Ostwald viscometer at a concentration of 0·05%.

	Moles of adenine/4P	Moles of guanine/4P	Moles of cytosine/4P	Moles of 5-methyl-cytosine/4P	Moles of thymine/4P
T_1 and T_2	1·02	0·75	0·78	0·07	1·01
H_1 and H_2	1·0	0·71	0·82	0·10	1·05
Wheat germ sample ...	0·90	0·66	0·65	0·19	0·91

0·05N- and 0·10N-Hydrochloric acid and potassium hydroxide were used to titrate solutions of initial volume 7—10 ml.

In order to determine the water correction, blank titrations against water of the acid and base used were performed as above.

Release of Phosphorus during the Dialysis of Nucleic Acids.—A solution of the sodium salt of thymus nucleic acid (T_1), containing sodium chloride (1·66M.), was analysed for nitrogen and phosphorus by the methods of Allen (*Biochem. J.*, 1940, **34**, 858) and of Ma and Zuazaga (*Ind. Eng. Chem., Anal.*, 1942, **14**, 280) respectively. The solution was dialysed at room temperature in cellophane tubes against running tap water until free from salts (1475 minutes) and then analysed as above. Dialysis caused only a 1% change in the N : P ratios and there was no significant change in pH.

In a further experiment a solution of the sodium salt of herring-roe nucleic acid, H_1 (200 mg.) in 1M-sodium chloride (125 ml.) was dialysed against distilled water (190 ml.), at room temperature. The dialysate was replaced by distilled water every 24 hours until free from sodium chloride. Phosphorus analysis of the combined dialysates showed that less than 1·6% of the total nucleic acid phosphorus originally present was released during the dialysis.

Release of Ammonia from Nucleic Acid Solutions at pH 12.—Potassium hydroxide solution (0·05M.) was added to a solution of nucleic acid (5 mg./ml.), until the pH was 12. Nitrogen was passed through the solution into Nessler's reagent, which remained unchanged in colour even after 30 minutes. Nucleic acid was shown not to interfere with the release of ammonia from the solution by adding ammonium chloride. Less than 0·1% of the nitrogen in the nucleic acid would have been detected by this method had it been released as ammonia.

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