743. *The Electrometric Titration of the Sodium Salts of Deoxyribonucleic Acids. Part II.* The Effect of Ultrasonic Irradiation.*

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The effects of ultrasonic irradiation on the titration curves of the sodium salt of the deoxyribonucleic acid of herring sperm have been studied. In the degraded product the difference between forward- and back-titration curves is greatly decreased, although the separation of the two back-titration curves still persists (see Part I *). There is an increase in the number of secondary phosphoryl groups and probably also in the number of primary phosphoryl groups. Ultrasonic waves cause a breakdown of hydrogen bonding and rupture of some internucleotide linkages.

It has been observed that high-frequency sound waves lower the viscosity and degrade both natural and synthetic polymers (Szent-Gyorgi, *Nature*, 1933, **131**, 278; Schmid and Rommel, *Z. physikal. Chem.*, 1940, *A*, **185**, 98; Melville and Murray, *Trans. Faraday Soc.*, 1950, **46**, 996). Recent work in this laboratory (Laland, Overend, and Stacey, *Research*, 1950, 386) and the investigation by Goldstein and Stern (*J. Polymer Sci.*, 1950, **5**, 687) have shown that deoxyribonucleic acids are similarly degraded by ultrasonic irradiation.

Laland, Overend, and Stacey (*loc. cit.*; also forthcoming publication) showed that ultrasonic irradiation of deoxyribonucleic acids, isolated from calf thymus glands, from soft herring roes, and from wheat germ, resulted in a rapid fall in viscosity which was not regained on storage but continued to fall slightly. During irradiation no purine or pyrimidine bases were liberated and only negligible amounts of inorganic phosphate and ammonia were formed. The products were still insoluble in acid and ethanol, but the structural viscosity and streaming birefringence of the original nucleic acid had disappeared. These and other physical measurements indicated that disaggregation had occurred but that the products were still of relatively high molecular weight, and this is confirmed by the sedimentation-constant measurements by Goldstein and Stern (*loc. cit.*).

Since certain features of the titration curves of nucleic acids can be explained by postulating various weak polymeric linkages (Part I, *loc. cit.*) likely to be susceptible to ultrasonic waves, the changes in the titration curves of herring sperm deoxyribonucleic acid degraded to different degrees by ultrasonic irradiation have been examined (for preliminary report see Overend and Peacocke, *Trans. Faraday Soc.*, 1950, **46**, 794). The results help to elucidate the nature of the breakdown due to the irradiation, and also confirm that the difference between forward- and back-titration curves, described in Part I, is closely associated with the existence of polymeric linkages, such as hydrogen bonds. That the separation of the curves obtained by back-titration with acid and alkali is hardly altered by ultrasonic degradation supports the hypothesis (see Part I) that this separation is due to an irreversible neutralisation of the alkali.

Titration Curves.—Figs. 1—3 show the titration curves of samples of the sodium salt of herring sperm deoxyribonucleic acid which have been isolated from solutions of the nucleic acid after various periods of ultrasonic irradiation. The irradiation was carried out at atmospheric pressure and under these conditions cavitation should occur, producing the maximum rate of depolymerisation. These figures should be compared with the titration curves of the same nucleic acid before irradiation (Fig. 2, Part I); the same Roman numerals are used to designate the various forward- and back-titration curves and the ordinate represents the same quantity as in the figures of Part I.

The main features of these titration curves are as follows. (*a*) As irradiation continues, the forward-titration curves approach the corresponding back-titration curves, and in the acid range the curves III and IV become almost coincident (Fig. 3). The area enclosed by the forward and back-titration curves is a measure of their relative displacement; the changes in these areas are given in Table I. Since certain reactions with alkali seem to affect the position of curve II (see Part I), the change is most clearly seen with curves III and IV. Unlike III and IV, curves I and II never become completely coincident, and a possible interpretation of this has already been discussed (Part I).

(*b*) The solutions given by irradiated nucleic acid samples were all of lower pH ($\simeq$5·5) than that given by the unirradiated nucleic acid (6·9). This indicates that some acidic group has been liberated. Goldstein and Stern (*loc. cit.*) observed no change in pH on irradiation for

* Part I, preceding paper.

TABLE I.

Ultrasonic irradiation time (mins.)	0	95	180	270
Area * enclosed by I and II	5·2	4·1	2·3	—
Area * enclosed by III and IV	3·0	1·7	1·4	0·2
Estimate † of increase in primary phosphoryl groups/4P {By II	—	0·12	0·12	0·20
{By IV	—	0·12	0·20	0·18
Estimate † of increase in secondary phosphoryl groups/4P {By II	—	0·20	0·16	0·36
{By IV	—	0·20	0·20	0·32
Total number of ‡ $(RO)_2PO\cdot ONa$ groups. By IV	2·84	2·44	2·44	2·20
Total number of secondary phosphoryl groups/4P. By IV	0·18	0·38	0·38	0·50
η (rel.) (25°) of nucleic acid solution before freeze-drying	5·8—6·5	1·8	1·33	1·21

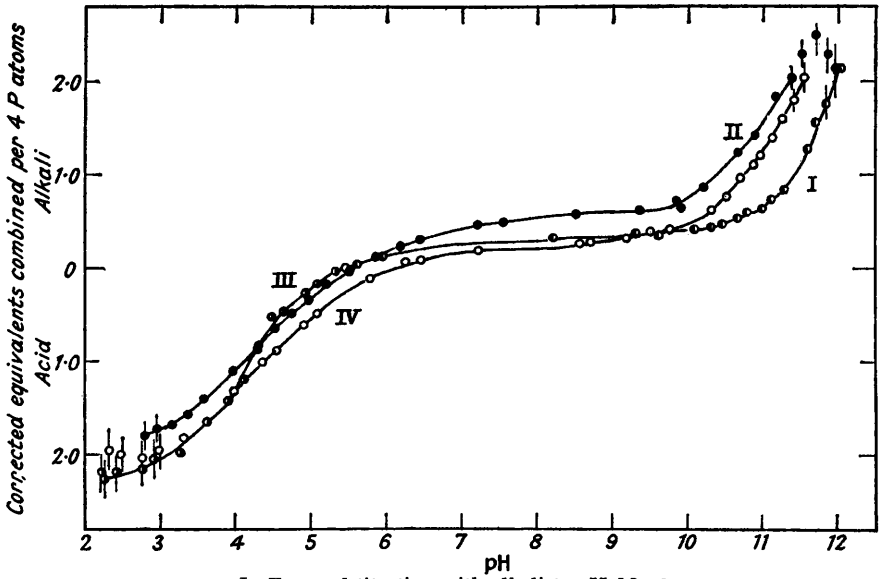
* Unit of area = rectangle formed by 1 unit on abscissa and 1 unit on ordinate of Fig. 1—3.

† The increase in the number of secondary phosphoryl groups is accurate only to within ± 0·06, and the increase in the primary groups is even less precisely measurable since it is sensitive not only to the error in each individual measurement but also to the absolute reproducibility of the curves.

‡ R = Nucleoside residue : calculated as *b* in Table II, Part I.

FIG. 1.

Titration curves of the sodium salt of herring sperm deoxyribonucleic acid after ultrasonic irradiation for 95 mins.



I. Forward-titration with alkali to pH 12, ◐.
II. Back-titration with acid from pH 12, ●.
III. Forward-titration with acid to pH 2, ◑.
IV. Back-titration with alkali from pH 2, ○.

periods of 25 minutes. The difference is probably caused by the much longer irradiation times employed in these experiments.

(*c*) Over the pH range 5—6, the titration curves of unirradiated nucleic acids rise more steeply than before irradiation. This characteristic of the curves is illustrated more precisely by Fig. 4, where a given curve, *e.g.*, II, of the unirradiated nucleic acid is compared with the corresponding curve (II) of an irradiated sample in order to eliminate the various effects of acid and alkali; the curves in Fig. 4 then represent changes due to the irradiation. The irradiation has caused an increase in the number of groups titrated over the pH range 5—6. Ammonia is formed in only negligible quantities by extended irradiation (Laland *et al.*, *loc. cit.*), so no deamination, with the appearance of xanthine hydroxyl groups ($pK' = 6{\cdot}0$ in xanthosine; Ogston, *J.*, 1935, 1376), can have occurred. The only other known groups dissociating near this region are the amino-groups of cytosine (dissociation constant in the ribonucleotide = 4·2; titration constant according to the analysis of Part I ≏4·85), and secondary phosphoryl groups (dissociation constants reported vary from 5·9 to 7·0 in ribonucleotides and sugar phosphates; for references see Part I). All the amino-groups of the bases, determined analytically, are free and titratable in curves II and IV obtained with the original nucleic acid (see Part I, where

the analytical figures for amino-groups can fully account for the titration curve in the acid region). The amino-groups are therefore not involved in any main covalent linkage in the unirradiated acid and there seems to be no possibility of an increase in their number. Finally,

FIG. 2.

Titration curves of the sodium salt of herring sperm deoxyribonucleic acid after ultrasonic irradiation for 180 min. Curves as in Fig. 1.

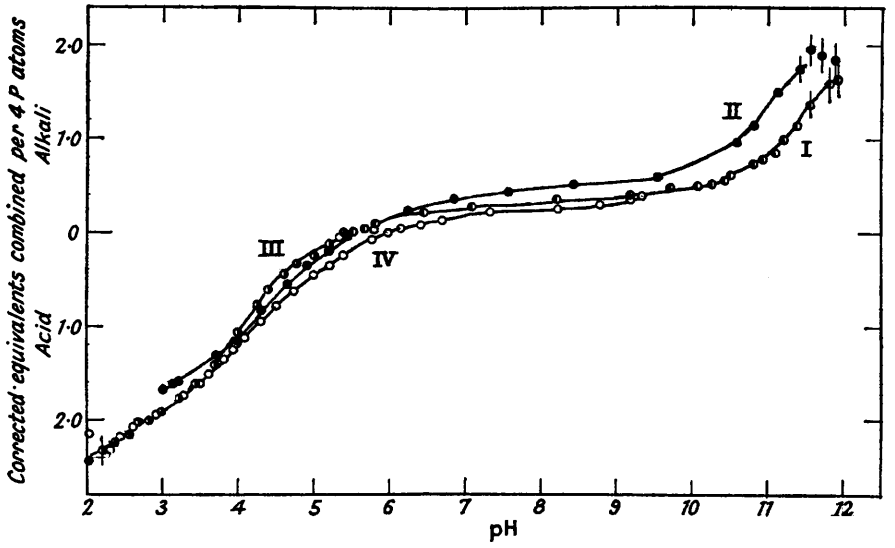
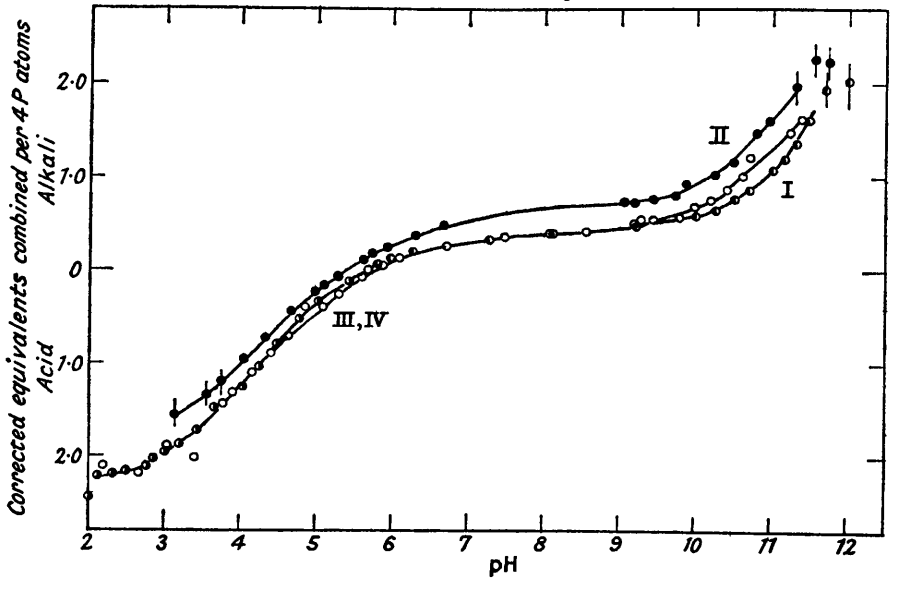


FIG. 3.

Titration curves of the sodium salt of herring sperm deoxyribonucleic acid after ultrasonic irradiation for 270 min. Curves as in Fig. 1.



any increase in the number of amino-groups would tend to increase the pH of the solution, not to decrease it, as observed. The extra groups formed which are titrated in the pH range 5—6 can therefore be identified as secondary phosphoryl groups.

The ordinate in Fig. 4 is finite and positive below pH 4, so the increase in the acidity of nucleic acid solutions after irradiation also seems to be partly caused by formation of acidic groups not titrated over the pH range (2—12) of the experiments. The only possible acidic group

fulfilling such conditions is the primary phosphoryl group. Table I records the approximate increase in the number of the two types of phosphoryl group. The figures for 270 minutes' irradiation indicate that even at this point, long after the viscosity has ceased to fall, secondary phosphoryl groups are still being formed.

(*d*) The separation of the back-titration curves II and IV, discussed in Part I, is also observed in these experiments, and Fig. 5 shows that it is very little affected by ultrasonic irradiation. This contrasts with the effect of irradiation on the displacement of a forward-titration curve

FIG. 4.

The effect of ultrasonic irradiation for various periods on the back-titration curves of the sodium salt of herring sperm deoxyribonucleic acid: difference between curves obtained on back-titration with alkali from pH 2, ○; *difference between curves obtained on back-titration with acid from* pH 12, ●.

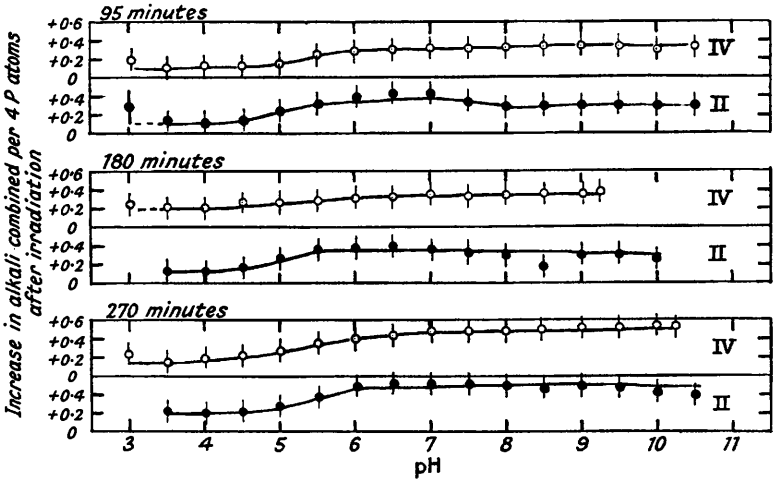
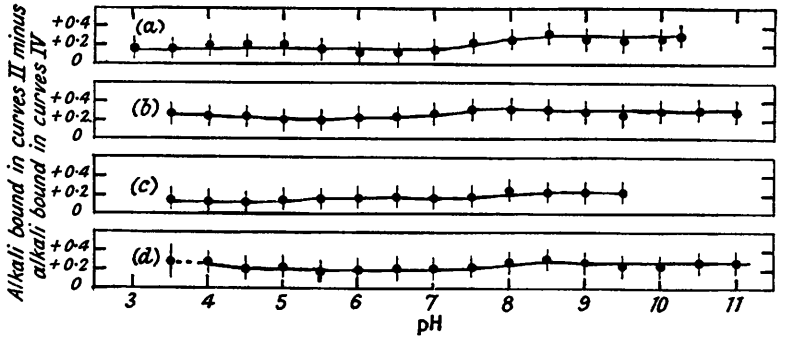


FIG. 5.

Difference between the two back-titration curves (II and IV) of the sodium salt of herring sperm deoxyribonucleic acid before and after ultrasonic irradiation. Irradiation time: (a) *Unirradiated*; (b) 95 *min.*; (c) 180 *min.*; (d) 270 *min.*



from the corresponding back-titration curve and different explanations of the two sets of observations are necessary (see Part I).

DISCUSSION.

The general conclusions of Laland, Overend, and Stacey (*loc. cit.*) are confirmed by the titration data presented here, since the main pattern of the curves is preserved and there is no major alteration in the number of groups titrated, excluding the phosphoryl groups. The titration data suggest two main effects of ultrasonic irradiation on deoxyribonucleic acids. First, the weaker bonds, which are responsible for the difference between forward- and back-titrations and are broken by mild acid or alkali treatment, are also gradually broken by continued irradiation. Gulland, Jordan, and Taylor (*J.*, 1947, 1131) and Creeth, Gulland, and Jordan (*ibid.*, p. 1141) have suggested that these weaker linkages are hydrogen bonds, cross-linking

groups in various polynucleotide chains, and that they are also responsible for the high structural viscosity of nucleic acid solutions. This suggestion is supported by the present work, which shows that ultrasonic irradiation decreases the displacement of the forward- from the back-titration curves and at the same time reduces the viscosity, incidentally destroying its structural character. The trends in these quantities as irradiation continues are expressed relatively to their initial values for the unirradiated nucleic acid in Table II. The viscosity is seen to decrease relatively faster than does the area enclosed by I and II and by III and IV.

TABLE II.

Time of irradiation (mins.)	0	95	180	270
Relative area enclosed by I and II	100	79	38—58	—
Relative area enclosed by III and IV	100	57	47	7
η (rel.) (25°) as % of original value before each irradiation	100·0	25·6	22·9	20·9

Secondly, ultrasonic irradiation causes an increase in the number of primary and secondary phosphoryl groups, and the latter groups are still being formed even after 270 minutes' irradiation, when the viscosity is altering very little. Groups of this type could only be formed by a gradual rupture of internucleotide linkages which are of a very much higher order of stability than the hydrogen bonds discussed above. This process must contribute to some extent to the fall in viscosity, although it would not be expected to be as important as the breakdown of hydrogen bonding in view of the very great decrease brought about by this factor alone during acid treatment (Creeth, Gulland, and Jordan, *loc. cit.*).

The formation of secondary phosphoryl groups when internucleotide linkages are broken is explicable on the basis of a branched or unbranched structure for the polynucleotide chain. However, new primary phosphoryl groups could only be formed as a result of the cleavage of bonds involving tri-esterified phosphoric acid residues. In Part I, the presence of such phosphorus atoms at not infrequent intervals along the main polynucleotide chain was suggested in order to explain the degree of branching implied by the titration data. The formation of primary phosphoryl groups on ultrasonic irradiation therefore supplies additional evidence for this hypothesis. Without further measurements of the size and shape of the fragments produced it is not possible to say whether ultrasonic irradiation breaks the main polynucleotide chain or removes the side branches or has a random effect. Melville and Murray's evidence (*loc. cit.*) that the weaker covalent linkages in synthetic copolymers are not preferentially ruptured by ultrasonic irradiation supports the latter alternative; they found no evidence for the formation of free radicals in their irradiations, which were carried out under conditions where the more effective cavitation could not occur. A free-radical mechanism also seems unlikely in these irradiations of nucleic acid under cavitation conditions, since there is no long-continued steep fall in viscosity after the irradiation has ceased (Laland, Overend, and Stacey, *loc. cit.*; contrast the action of *X*-irradiation in the presence of oxygen, Butler and Conway, *J.*, 1950, 3418).

EXPERIMENTAL.

The sodium salt of herring sperm deoxyribonucleic acid used in these experiments was the sample H_1 of Part I.

The sonic oscillator was that described by Melville and Murray (*loc. cit.*). Aqueous solutions of H_1 (0·2 g./100 ml.) were irradiated at atmospheric pressure for periods of 95, 180, and 270 minutes and were surrounded by a cooling tube which kept the temperature at about 20°. The anode current was kept constant at a value of 70 m. amp. and this ensured that the intensity was the same in all experiments After irradiation, the viscosity of the solutions was determined (see Table I), and the degraded nucleic acid was isolated by drying in the frozen state.

The nucleic acid solutions were titrated at 25° under a nitrogen atmosphere, and the pH's of the solutions were determined by means of a glass electrode and a saturated calomel half-cell. The concentrations of the solutions used, the method of calibrating the glass electrodes, and the calculations of acid and base combined were the same as those described in Part I. The error in each individual pH measurement was not greater than $\pm$0·01 unit, and the calibration of the electrodes was frequently checked during and after the titrations.

All viscosity measurements were made at 25° in an Ostwald viscometer; η(rel.) in Table I is the ratio of the flow time of 5 ml. of the nucleic acid solution (0·2 g./100 ml.) to the flow time of 5 ml. of distilled water.

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