THE ULTRAVIOLET ABSORPTION OF SOME DEGRADED DESOXYRIBONUCLEIC ACIDS

by

S. G. LALAND*, W. A. LEE, W. G. OVEREND AND A. R. PEACOCKE
Chemistry Department, University of Birmingham (England)

It has been the purpose of many studies[1–19] on sodium desoxyribonucleates (DNA) to obtain data which could be used to support a definition of the state of aggregation and the molecular size and shape of nucleic acids of this type. With this aim in view, we have been interested in recent years in the degradation of nucleic acids by physical methods[20], and we have studied the degraded products so prepared by the electrometric titration technique[21]. It is well known that when calf thymus DNA is degraded enzymically by pancreatic desoxyribonuclease, there is an increase in the intensity of the ultraviolet absorption exhibited by the nucleic acid[22]. Similar effects have been observed when the nucleic acid is degraded in other ways[11]. In order to study further the relationship between the degree of polymerisation of DNA's and their ultraviolet absorption spectra, we have investigated the spectral changes which result when DNA isolated from either calf thymus gland, soft herring roes, wheat germ or *M. phlei* is degraded with desoxyribonuclease, by ultrasonic waves, or thermally. Furthermore, the concomitant spectral changes accompanying the electrometric titration of DNA from herring sperm have been studied.

EXPERIMENTAL STUDIES

Materials

DNA's were isolated from calf thymus gland, soft herring roes, wheat germ and *M. Phlei* according to the methods already described by some of us[23], and the materials had essentially the properties and composition as described in the original publication[23]. The DNA used in the electrometric titration studies was prepared by obtaining a solution of desoxypentose nucleoprotein from fresh herring sperm by the method of MIRSKY AND POLLISTER[24]. The sodium salt of the desoxypentose nucleic acid was isolated from this by precipitation of the protein with excess sodium chloride. Centrifugation, followed by removal of the last traces of protein by the procedure described by SEVAG *et al.*[25] and precipitation by addition of ethanol, afforded the DNA, which was washed with 90% ethanol and then dried in the frozen state. (Found: N, 13.83; P, 9.10%; N/P (%) = 1.52; Na/P (atomic) 0.95 ± 0.01). The sample contained 0.3% of sodium ribonucleate and had the following composition.

Base	Adenine	Guanine	Cytosine	5-Methyl Cytosine	Thymine
Moles/4 moles P	1.09	0.85	0.80	0.06	1.05

Molecular weight determinations by light-scattering measurements using the apparatus of Dr.

* Present address, Institute for Nutrition Research, University of Oslo, Norway.

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S. KATZ (see 26) indicated that the substance had a molecular weight of $4.6 \cdot 10^6$. It showed λ_{max} at 259 mμ, the $\varepsilon_{(p)}$ in the absence of electrolyte being 7200 (average of 6 independent determinations varying by ± 2%).

Degradation of nucleic acids

a. *By ultrasonic waves*

DNA's isolated from calf thymus glands, soft herring roes and wheat germ were separately degraded by ultrasonic waves according to the procedure described by LALAND, OVEREND AND STACEY[20]. The values obtained for the absorption at 259 mμ (*i.e.* wavelength of maximum absorption) by the nucleic acids in solution in distilled water, before and after treatment are shown in Table I. (The values $[\varepsilon_{(p)259}]$ reported are atomic extinction coefficients with respect to the phosphorus contents of the nucleic acids, *cf.* CHARGAFF AND ZAMENHOF[27].) The absorptions were measured with a "Unicam" spectrophotometer. In addition, the original and final pH values of the solutions are recorded (Cambridge pH meter).

TABLE I

SOME EFFECTS OF ULTRASONIC IRRADIATION ON NUCLEIC ACID

Source of DNA	*pH of solution*		$\varepsilon_{(p)259}$	
	Before irradiation	*After irradiation*	*Before irradiation*	*After irradiation*
Calf thymus gland	5.62	5.00	7700	7800
Soft herring roes	6.06	5.50	7500	7620
Wheat germ	5.90	5.46	7300	7400

It appears that the degradation of DNA by ultrasonic waves, as indicated by the drop in viscosity of the solution (see LALAND, OVEREND AND STACEY[20]) is accompanied by only a very slight change in the intensity of absorption of U.V. light.

b. *By desoxyribonuclease*

The general method adopted was as follows: DNA in aqueous solution (10 ml) (0.2% solution in the case of calf thymus DNA and a 0.4% solution for the other samples of DNA), and phosphate buffer (pH 7, 0.1 *M*, 5 ml) containing magnesium sulphate (0.1 *M*) were mixed and maintained at 37° for 10 minutes. Thereafter desoxyribonuclease isolated according to the procedure of MCCARTY[28] (5 ml of a 0.002% solution) was added, and an aliquot (5 ml) was immediately transferred to an Ostwald viscometer in a thermostat at 37°. Changes in viscosity (as measured by time of flow) and ultraviolet absorption were measured at noted intervals of time. The light absorption measurements were performed by diluting a portion (0.25 ml, except in the case of calf thymus DNA, when 0.5 ml was used) of the digest with distilled water (final volume was 10 ml) and taking measurements on this and comparing with controls containing the same amount of enzyme and inorganic ions, but no DNA. Measurements of light absorption were carried out with a "Unicam" spectrophotometer at wavelengths of 230, 259 and 275 mμ. Initial measurements of the ultraviolet absorption and viscosity were made one minute after mixing of substrate and enzyme solutions: the results so obtained were used as zero values. Results are shown in Table II (a–d). In each case a calculation was made

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TABLE II

CHANGES IN VISCOSITY AND LIGHT ABSORPTION ON TREATMENT OF DNA SOLUTIONS WITH DESOXYRIBONUCLEASE

Calf thymus DNA[a]	Time of digestion (Minutes)	1	5	13	25	38	58	65	88	128	158	720
	Change in viscosity (% of total change)	0	47	68.5	84	93	96	—	100	100	100	100
	Change in absorption at 259 mμ (% of total change)	0	0	0	0	0	—	11.8	27.5	59	82	100
Herring roe DNA[b]	Time of digestion (Minutes)	1	12	16	27	50	97	174	207	312		
	Change in viscosity (% of total change)	0	8.4	10.7	20.5	36.5	56.5	79	100	100		
	Change in absorption at 259 mμ (% of total change)	0	0	0	0	0	0	—	13.3	63.5		
Wheat germ DNA[c]	Time of digestion (Minutes)	1	2	8	13	18	138					
	Change in viscosity (% of total change)	0	0	100	—	—	—					
	Change in absorption at 259 mμ (% of total change)	0	0	39.2	65.0	78	100					
M. phlei DNA[d]	Time of digestion (Minutes)	1	5	10	20	30	75	160	228			
	Change in viscosity (% of total change)	0	80	100	—	—	—	—	—			
	Change in absorption at 259 mμ (% of total change)	0	0	16.8	25	44	54	65	100			

The initial and final values of $\varepsilon_{(p)\ 259}$ before and after enzymic treatment respectively were (a) 7700 and 10,130, (b) 7500 and 10,320, (c)7300 and 9420 and (d) 6750 and 9020.

of the percentage increase in light absorption at various wavelengths, by the digest after incubation for 15 hours. Table III shows the results obtained.

TABLE III

PERCENTAGE INCREASE IN LIGHT ABSORPTION AT VARIOUS WAVELENGTHS BY DNA'S AFTER TREATMENT WITH DESOXYRIBONUCLEASE

Source of DNA	*Percentage increase in light absorption*		
	230 mμ	*259 mμ*	*275 mμ*
Calf thymus gland	28.0	31.5	33.5
Soft herring roes	43.5	37.5	46.5
Wheat germ	38.7	29.0	38.0
M. phlei	45.5	33.6	52.0

From the above measurements on the changes in viscosity and light absorption on degradation of DNA's by desoxyribonuclease, it is apparent that the increase in light absorption does not begin until the decrease in viscosity is essentially complete. This is particularly so for DNA isolated from calf thymus gland and soft herring roes. With DNA isolated from wheat germ and *M. phlei* the lag period before the onset of increase in light absorption is much reduced, but this is probably due to the somewhat degraded nature of these nucleic acids, due to difficulties encountered in their isolation. The increase in the degree of light absorption by DNA, when acted on by desoxyribonuclease, takes place over a range of wavelengths. At a wavelength of 259 mμ the increase in absorption for all samples of nucleic acid examined was in the region of 30–35%.

c. *By heat treatment*

Calf thymus DNA (20 mg) was dissolved in distilled water (20 ml) and viscosity measurements (Ostwald viscometer at 37°) and light absorption readings were taken. The solution was heated at 100° for 45 minutes and viscosity and light absorption measurements were re-taken. The initial relative viscosity value of 10.0 decreased during the heating to a final value of 1.9. The corresponding increase in light absorption at 259 mμ was 24% of the initial absorption. In a subsequent experiment calf thymus DNA (30 mg) in solution in distilled water (30 ml) was heated at 100° for 20 minutes. The above measurements were repeated and in addition pH measurements (Cambridge pH meter) were taken. In this case the initial relative viscosity value of 14 changed to 4.0 after this period of heating, and simultaneously the increase in light absorption at 259 mμ was 21% of the initial absorption. The pH values of the solution before and after heating were 6.02 and 6.12 respectively.

It is apparent from these results that thermal degradation of DNA solutions is accompanied by an increase in light absorption properties of the solutions, but not by any significant increase in the pH value (*cf.* GOLDSTEIN AND STERN[11]).

d. *Acid and alkali treatment*

In connection with a related study on the electrometric titration of DNA at different ionic strengths[29], solutions of herring sperm DNA (0.2%, moist basis, 0.141 mg P/ml) containing varying amounts of sodium chloride, hydrochloric acid and/or sodium

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hydroxide (to give solutions of constant total uni-univalent electrolyte concentrations) were prepared. The concentration of free hydrogen ions in these various mixtures was determined at 25° in a small (1.8 ml) Clark-type electrode cell[30] containing two hydrogen and two silver-silver chloride electrodes without a liquid junction. The E.M.F. of the cell became steady after a few minutes except with solutions the final pH of which was greater than 10.5: with these the E.M.F. drifted slowly in a direction which indicated a slow neutralisation of alkali (*cf.* reference 21). The E.M.F.'s given by blank solutions, omitting the nucleate, were measured and enabled the hydrogen ions dissociated or bound per 4 g atoms of nucleate phosphorus to be calculated. The back-titration curve from *ca* pH 2.5 was obtained by adding the reagents in the order given and from *ca* pH 12 by adding alkali before acid; there was about half a minute between these additions thereby minimising alkaline hydrolysis effects. The characteristic difference, attributed to the presence of hydrogen bonds[21,31], between the titration curves before and after acid or alkali titration treatment was observed at all ionic strengths. Under the conditions of the experiments, in particular the presence of salt during the addition of acid and alkali, the discrepancy between the titration curves before and after acid treatment was smaller at the higher ionic strengths. This was the result of a combination of various factors, but it implied that less hydrogen bonds were broken by a given lowering of pH at the higher ionic strengths[29].

Parallel with these titration studies an aliquot (0.5 ml) of the titration mixture was diluted with distilled water (20 ml) and the extinction coefficient of the resulting solution was measured at $\lambda = 230$–290 mμ in a "Unicam" spectrophotometer using 1 cm cells. A solution of identical composition in all electrolytes, except that the DNA was omitted, was used as the reference blank for each diluted mixture. In Fig. 1, the values of the extinction coefficients at 259 mμ, so obtained, are plotted against the negative logarithm of the free hydrogen ion concentration (pH_c) of the titration solutions before dilution for light absorption measurements. Dilution will modify the pH but will

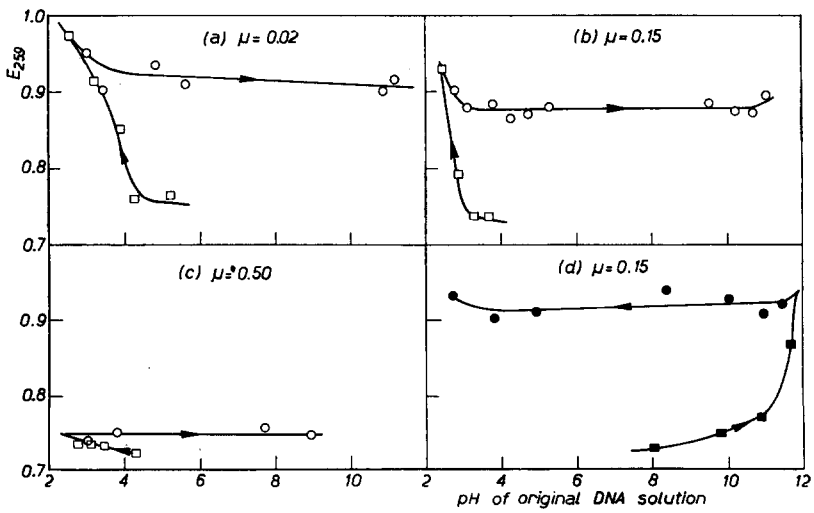

Fig. 1. The effect of acid and of alkali treatment on the ultraviolet absorption at 259 mμ of herring sperm DNA. Concentration of DNA = 0.00344 mg P/ml. pH_c – see experimental section; Squares – forward titrations; circles – back titrations; □, ○, acid treatment; ■, ●, alkali treatment. Total non-nucleate ionic strengths (μ): (a) 0.02, (b) 0.15, (c) 0.50, (d) 0.15.

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not alter the sequence of the points. The curves show that mild acid or alkali treatment under the conditions described (25°, and less than 0.5 minute between the additions of alkali and acid) causes an increase in the extinction coefficient which is only partly reversed on back titration. The increase is almost negligible under conditions (Curve c, acid treatment, non-nucleate ionic strength = 0.50) where the titration evidence already suggests that fewer hydrogen bonds have been broken by the treatment (*cf.* LEE AND PEACOCKE[29], Fig. 1). The full extinction curves showed that the greatest change in ultraviolet absorption due to acid or alkali treatment was in the region of 260 mμ, although there were also smaller changes at other wavelengths, and that the wavelength of maximum absorption remained in the 258–260 mμ region.

DISCUSSION OF RESULTS

In a previous communication[20] evidence was presented which indicates that during ultrasonic irradiation of herring sperm DNA (sodium salt), the viscosity rapidly decreases to a fraction of its initial value and the DNA loses its structural non-Newtonian character. After 95 minutes irradiation under the conditions described, the change becomes very much slower, the viscosity relative to water decreasing from 1.8 to 1.21 in a further 175 minutes. On electrometric titration of the nucleate after irradiation for 95 minutes it was found that the difference between the forward and back-titration curves persisted, although it was somewhat reduced (LEE AND PEACOCKE[21]). Considerable hydrogen bonding therefore remains although the viscosity changes indicated that there had been a marked decrease in the interaction between different polynucleotide chains. It seems probable that during the irradiation (for 95 minutes) intermolecular interactions (Van der Waals, electrostatic and possibly hydrogen bonding) are reduced, and that the remaining hydrogen bonds, as indicated by the titration studies, must link chiefly groups within the same chain. This is not surprising considering the nature of the shearing forces resulting from ultrasonic waves. During this period of irradiation, the extinction coefficient (calculated with respect to the phosphorus content) at 259 mμ ($\varepsilon_{(p)259}$) only changes from 7500 to 7620, thereby implying that the absorption is not significantly altered by reduction of intermolecular interactions.

The nature of the intramolecular hydrogen bonds has been elucidated in recent structures for DNA based on the fibre X-ray patterns[32,33]. These structures contain inter-twined helical polynucleotide chains, with the flat purine and pyrimidine rings set in planes almost perpendicular to the main axis of the helices and superimposed in slightly offset positions at a distance along the axis direction of 3.4 A. Hydrogen bonds cross link the two helical chains through titratable groups of base molecules directed inwards, and rupture of these bonds would be expected to lead to a breakdown of the whole molecular arrangement. Since these hydrogen bonds are concerned so intimately in linking groups within the molecular unit, and since their rupture would not lead necessarily to a drop in the viscosity of a nucleic acid solution, they may be regarded as "intramolecular".

Prolonged ultrasonic irradiation must break the intramolecular hydrogen bonds, since after treatment for 270 minutes, the discrepancy between the forward and back titration curves, at least below pH 7, has almost disappeared[21]. At all stages of the irradiation some internucleotide linkages can be shown to have been ruptured, but

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apparently without much effect initially on the ultraviolet absorption spectrum of the DNA.

A similar interpretation must apply to the effects of ultrasonic irradiation on calf thymus DNA and wheat germ DNA (sodium salts). In these cases also, there is no significant change in $\varepsilon_{(p)\ 259}$ during the periods (240 and 95 minutes respectively) in which most of the decrease in viscosity occurs (*cf.* Ref. 20). Irradiation with sonic waves of calf thymus DNA for a similarly defined period appears to result in changes which parallel those obtained on ultrasonic irradiation, since there is little change in the ultraviolet absorption spectrum[11] and the rupture of internucleotide linkages is indicated by a decrease in molecular weight from $4\text{–}8 \cdot 10^6$ (*cf.* Ref. 34) to a value of the order of 300,000[35].

It is therefore our opinion that during the initial stages of sonic and ultrasonic irradiation of DNA there is a decrease in the interaction of different polynucleotide chains with no effect on the ultraviolet absorption of the material. A similar phenomenon occurs in the initial stages of desoxyribonuclease action on DNA and the explanation must be of the same type. For example, during the first stage of degradation of DNA by pancreatic desoxyribonuclease there is a marked decrease in the viscosity of the solution, a steep fall in the value of the dielectric constant, but no change in acid-precipitability[36] and little change in $\varepsilon_{(p)\ 258\text{—}260}$. This stage, which has been interpreted as a disaggregation[36,37], may involve formation of a complex between the enzyme and the "outside" of the nucleic acid helices. The main increase in optical density occurs when the viscosity is changing only very slowly, if at all, and when the DNA molecule is being degraded into acid-soluble dialysable fragments. During this latter stage intramolecular hydrogen bonds will be broken presumably, but an increase in the extinction will also arise from the breakdown of the DNA to oligonucleotides and smaller molecules (see below and refs. 39, 40) and so the effect is composite.

Section (d) of the experimental section shows that as groups in DNA are released for titration by addition of acid or alkali, there is a corresponding increase in the values of $\varepsilon_{(p)\ 259}$, and this increase is only partly reversed on back-titration. The increase in absorption is small (see Fig. 1) when the discrepancy between the forward and back-titration curves is also small, as for example, during the titration at an ionic strength of 0.50. Hence, it is exceedingly probable that change in the same structural feature is responsible for both the increase in extinction coefficient and displacement of the back from the forward titration curves. This change has been described[31] as the rupture of hydrogen bonds linking titratable groups, and this description is now supported by the X-ray evidence. On addition of alkali, the increase in ultraviolet absorption of the nucleic acid, consequent on the rupture of hydrogen bonds, must be overwhelming the decrease (at 259 mμ) which would be expected for desoxypentose nucleates, from the behaviour in alkaline solution of mixtures of purines and pyrimidines in the appropriate proportions[38,41,42]. Similar effects of mild acid and alkali treatment on the ultraviolet absorption of desoxypentose nucleates have been observed by other workers[38,43,44]. As changes in viscosity due to such treatment may be attributed to a variety of causes (*cf.* refs. 31, 45–47), no direct correlation is to be expected between this effect and change in extinction coefficient.

More drastic acid and alkali treatments, under conditions which must have resulted in considerable rupture of internucleotide linkages, have been shown to cause irreversible increases of 25 to 40% in the $\varepsilon_{258\text{—}260}$ of mouse liver nucleates (DNA and RNA)[39] and

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of ribonucleic acids (RNA)[40]. Since there is no anomalous titration evidence for hydrogen bonding in RNA's as prepared at present, it would seem that even in situations where no hydrogen bonding appears to be present, degradation to molecules containing only a few nucleotides is accompanied by an increase in the extinction coefficient to a value equal to that of a mixture of mononucleotides of the same composition. This effect is additional to the increase in $\varepsilon_{258-260}$ associated with hydrogen bonding, with which this paper is chiefly concerned.

When aqueous solutions of calf thymus DNA were heated at 100° for periods of 20 to 45 minutes there was a large decrease in viscosity accompanied by an increase in $\varepsilon_{(p)\ 258-260}$ (see also THOMAS[44], and KURNICK[48]). Similar observations were made by GOLDSTEIN AND STERN[11] who suggested that the increase in optical density was due to a change in pH, which, however, was not recorded. In the experiments reported in the present paper, no significant change in pH was observed, and a more likely explanation of the increases in absorption is to be found in the rupture of intramolecular hydrogen bonds. It has been reported[19,49] that ionizable groups are liberated when DNA solutions are heated, so that the titration curve is similar to the back-titration curves obtained after acid or alkali treatment. This indicates that heat treatment, in addition to any effects on intermolecular interactions, causes rupture of intramolecular hydrogen bonds which results in an increase in optical density.

The question arises as to the mechanism whereby rupture of intramolecular hydrogen bonds in DNA can result in a change in the optical density of its solution. It is impossible to give any definite answer, but molecular arrangements whereby aromatic rings are superimposed in layers above each other in a direction normal to the plane of the rings (*e.g.* the dye *pseudo-iso*-cyanine[50]) have been shown to have an absorption spectrum different from more random arrangements, and many dyes show a weakening of the main absorption band when interaction occurs in concentrated solutions[51]. This appears to be the result of overlapping of π-orbitals in adjacent rings with the formation of some type of low energy molecular bond (see DEWAR[52]). Since in the postulated helical structure[33] for DNA the flat base rings are closely set above each other (*i.e.* along the direction of the helical axis) in slightly offset positions, it is conceivable that breakdown of this structure by rupture of intramolecular hydrogen bonds might bring about a change in absorption spectrum by destroying π-bond interaction.

In conclusion, it would seem advisable to avoid correlating too closely the value of $\varepsilon_{(p)}$ with the degree of polymerisation of the DNA molecule (*cf.* the initial enzymic disaggregation of DNA without concomitant change in $\varepsilon_{(p)260}$). Moreover, deductions concerning the amounts of nucleic acid present in cells, based on their ultraviolet absorption can be misleading since this absorption depends very much on the state of the nucleic acid as it is present in the cell.

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SUMMARY

Measurements have been made of the atomic extinction coefficient with respect to phosphorus, at 259 mμ of various samples of degraded DNA. The nucleic acids used were isolated from animal, plant and microbial sources and were degraded by acid and alkali, ultrasonic waves, an enzyme,

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and by heat treatment. The thesis is developed that treatment which results in a decrease in intermolecular forces does not affect the absorption spectrum, whereas scission of intramolecular hydrogen bonds results in an increase in the optical density of the DNA solution.

RÉSUMÉ

Les coefficients atomiques d'extinction par rapport au phosphore dans divers spécimens d'acide désoxyribonucléique dégradé ont été mesurés à une longueur d'onde de 259 mμ. Les acides nucléiques utilisés ont été isolés de sources animales, végétales et microbiennes, et dégradés par l'acide, l'alcali, les ondes ultrasonores, un enzyme ou la chaleur. On a émis la théorie que la diminution des forces intermoléculaires est sans effet sur le spectre d'absorption, tandis que celle des liaisons hydrogène intramoléculaires fait diminuer la transparence de la solution.

ZUSAMMENFASSUNG

Die atomischen Extinktionskoeffizienten des Phosphors in verschiedenen Proben von abgebauten Desoxyribonukleinsäuren sind bei 259 mμ gemessen worden. Die Nukleinsäuren sind aus Tieren, Pflanzen und Mikroben isoliert, und mit Säure, Alkali, Ultraschallwellen, Enzym oder Hitze abgebaut worden. Aus den Beobachtungen folgt die Theorie, dass eine Behandlung, welche eine Schwächung der intermolekularen Bindungen bewirkt, das Absorptionsspektrum nicht beeinflusst, während die Spaltung von intramolekularen Wasserstoffbindungen die Durchsichtigkeit der Lösung vermindert.

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Note added to Proof

Since this paper was prepared for publication several authors have reported that the ultraviolet absorption at 258–260 mμ of aqueous DNA solutions is decreased by additions of small amounts of various salts (*cf.* J. SHACK, R. J. JENKINS AND J. M. THOMPSETT, *J. Biol. Chem.*, 203 (1953) 373; E. R. BLOUT AND A. ASADOURIAN, *Biochim. Biophys. Acta*, 13 (1954) 455; R. THOMAS, *Trans. Faraday Soc.*, 50 (1954) 304). This effect would appear to be the converse of that reported above and to involve a contraction of the polynucleotide chains which brings purine and pyrimidine rings nearer to each other, in contrast to the effect of agents which separate the rings by rupture of hydrogen bonds. Titration evidence (ref. 29 and unpublished observations) has already shown that the hydrogen bonded structure is present even in 0.50 *M* sodium chloride solution.