

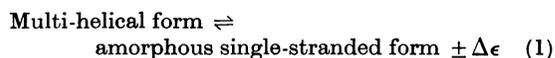
A Spectrophotometric Study of the Denaturation of Deoxyribonucleic Acid in the Presence of Urea or Formaldehyde and its Relevance to the Secondary Structure of Single-Stranded Polynucleotides

By R. A. COX AND K. KANAGALINGAM
National Institute for Medical Research, London, N.W. 7

(Received 28 November 1967)

1. The thermal denaturation of DNA from rat liver was studied spectrophotometrically. In sodium phosphate buffers denaturation led to a single-stranded form having, at 25°, about 25% of the hypochromism of the intact double helix. 2. The hypochromism of the denatured form was the same in 1mM- as in 10mM-sodium phosphate buffer and was scarcely affected by reaction with formaldehyde. The hypochromism was decreased by about 40% in the presence of 8M-urea. 3. The hypochromism of denatured DNA at low ionic strengths was about the same as that of fragments of reticulocyte ribosomal RNA that were too short to form double-helical secondary structure and about the same as that of RNA after reaction with formaldehyde. 4. The spectrum of DNA was slightly affected by the presence of 8M-urea or 4M-guanidinium chloride. The differences in the spectrum of the native and denatured forms of DNA in 0.1M-sodium phosphate buffer, in 8M-urea-10mM-sodium phosphate buffer and in 4M-guanidinium chloride-10mM-sodium phosphate buffer, pH 7.6, were similar but not identical. 5. Denatured rat liver DNA appears to have no double-helical character at 25° in 10mM-sodium phosphate buffer, pH 7.6; increasing the buffer concentration to 0.1M leads to a more compact form in which about 40% of the residues form base pairs.

When high-molecular-weight polynucleotides such as native DNA are heated in neutral salt solutions the transition to the component single strands is vividly marked by an abrupt change in the spectrum (below 300m μ), which takes place over a narrow temperature range. The transition is often regarded as:



The polynucleotide is considered to be amorphous when the base residues have no preferred orientation. However, a single-stranded polynucleotide is rarely amorphous, though this state is often approached at 95°. Thus eqn. (1) is a useful approximation when denaturation takes place at about 95°. When eqn. (1) applies the difference in the spectra of well-characterized multi-helical structures accurately reflects the nucleotide composition of the species undergoing the transition; for example, the nucleotide composition of DNA may be deduced from its denaturation spectrum (Felsenfeld & Sandeen, 1962; Mahler, Kline & Mehrotra, 1964; Hirschman & Felsenfeld, 1966).

The correlation between the nucleotide composition and the denaturation spectrum has been

exploited (Felsenfeld & Sandeen, 1962; Fresco, Klotz & Richards, 1963; Felsenfeld & Cantoni, 1964; Cox, 1966*a,b*; Guschlbauer, 1966) in studies of the secondary structure of single-stranded polynucleotides such as ribosomal RNA or denatured DNA. In these cases double-helical and single-stranded regions alternate. Unpaired residues in single-stranded regions have a tendency to 'stack' one upon another (for reviews see Felsenfeld & Miles, 1967; Michelson, Massoulié & Guschlbauer, 1967), the u.v.-absorption spectrum is affected and hypochromism is observed. As the temperature is raised the proportion of base-paired and of 'stacked' but unpaired residues is altered, so that the difference spectrum is the sum of both effects and the contribution of unpaired residues cannot be neglected (Cox & Kanagalingam, 1967*b*).

Previously, an estimate of the hypochromism due to double-helical structure was obtained in two ways (Cox & Kanagalingam, 1967*b*). First, the hypochromism of base residues in single-stranded regions was preferentially diminished through the use of reagents such as 4M-guanidinium chloride, which appear to decrease hypochromism due to stacking without affecting the hypochromism of double-helical structures. Secondly, allowance

was made for the hypochromism of 'stacked' residues by a procedure that requires a knowledge of the hypochromism of the entirely single-stranded polynucleotide. For this purpose RNA was treated with formaldehyde to prevent the formation of base pairs. It was assumed that chemical modification of the base residues did not affect hypochromism due to 'stacking'.

The aim of the present work was to study more closely the methods used to estimate the contribution of residues in single- and double-stranded regions to hypochromism. The effects of 8M-urea and 4M-guanidinium chloride on both the thermal stability and the denaturation spectrum of rat liver DNA were therefore examined. Moreover, the hypochromism of formaldehyde-treated DNA was compared with that of denatured DNA at low ionic strengths, when double-helical structure is absent. The hypochromism of formaldehyde-treated RNA was also compared with the hypochromism of fragments of RNA that are too small to form intramolecular double-helical structures. The results provide information about the conformation of denatured DNA at high and low ionic strengths and also permit the hypochromism of single-stranded polyribonucleotides and polydeoxyribonucleotides to be compared.

EXPERIMENTAL

Materials. DNA (which was given by G. R. Wyatt) had been isolated by the procedure of Marmur (1961) from rat liver nuclei, prepared by the method of Widnell & Tata (1964). RNA was removed by digestion with ribonuclease, and traces of protein were hydrolysed with Pronase. The DNA sample (0.05g./l.) was found to sediment as a single boundary in 0.1M-sodium phosphate buffer, pH 7.0, with $S_{20,w}$ 21.6s. Calf thymus DNA was isolated by the method of Kay & Dounce (1953).

RNA was isolated from the fractionated sub-particles of rabbit reticulocyte ribosomes. Rabbits were made anaemic by five daily injections of phenylhydrazine. Blood was obtained by heart puncture and reticulocyte ribosomes were isolated by the method described by Arnstein, Cox & Hunt (1964). The ribosomal sub-particles were prepared by titration with EDTA (Gould, Arnstein & Cox, 1966) and fractionated by zone centrifuging (Klucis & Gould, 1966). RNA was isolated by precipitation as the guanidinium salt (Cox, 1966c).

Chemicals. Guanidinium chloride (6M) was prepared by adding A.R. guanidinium carbonate (108g.) to conc. HCl (100ml). The solution was brought to pH 7, CO₂ was expelled by heating to about 60° and the solution was made up to 200ml. After addition of Norit charcoal to remove u.v.-absorbing impurities and filtration, the solution was heated for 5 min. at 95°. The solution was then transparent at 260m μ and remained transparent on heating slowly to 95°. A.R. urea was recrystallized from aqueous ethanol. Other reagents, formalin and sodium phosphate were of A.R. quality and were used without further purification.

Methods. Extinction measurements were made with a Unicam SP.700 spectrophotometer. The sample cell holder was an electrically heated copper block the temperature of which was controlled to within $\pm 0.1^\circ$ at any temperature in the range 25–95°. An allowance was made for changes in extinction due to changes in concentration arising from the thermal expansion of water.

Measurements of viscosity were made with an Ostwald-Fenske viscometer (Canon Instrument Co., State College, Pa., U.S.A) requiring 10ml. and having a flow time for water of 245.3 sec. at 25°. The temperature was maintained by means of a thermostatically controlled water bath at either 25° or 55°.

A Spinco model E ultracentrifuge fitted with ultraviolet optics was used to measure sedimentation coefficients. The temperature was controlled at 18–20° and the extinction of the solution was scanned at 4 min. intervals after reaching full speed (42040 rev./min.).

RESULTS

Hypochromism of denatured DNA in 1mM- and 10mM-sodium phosphate buffer, pH 7.6. A stock solution of rat liver DNA (5mg./ml.) in 0.15M-sodium chloride–15mM-sodium citrate buffer,

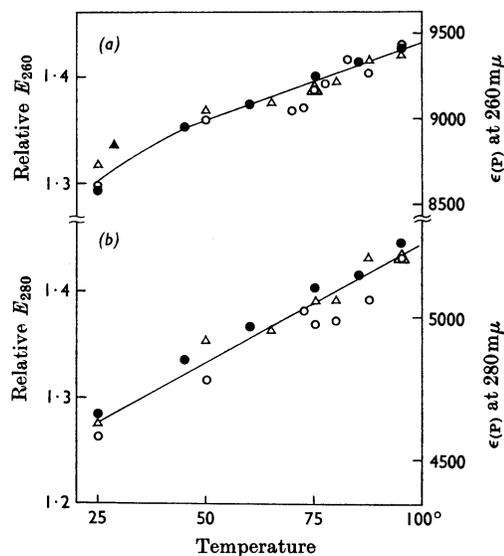


Fig. 1. Dependence of the extinction of denatured rat liver DNA on temperature. Rat liver DNA was dissolved in buffer and heated to 95° (see Fig. 2). The solution was cooled quickly and the values of extinction (relative to those observed for native DNA at 25°) obtained on heating for the second time are given in the Figure: O, solvent 10mM-sodium phosphate buffer, pH 7.6; ●, solvent 1mM-sodium phosphate buffer, pH 7.6; Δ, solvent 1% formaldehyde–0.1M-sodium phosphate buffer, pH 7.6, previously heated to 95° (in this case 800 was subtracted from each value of ϵ_{260} and 2100 was subtracted from each value of ϵ_{280} to facilitate comparison with the values obtained in phosphate buffer alone).

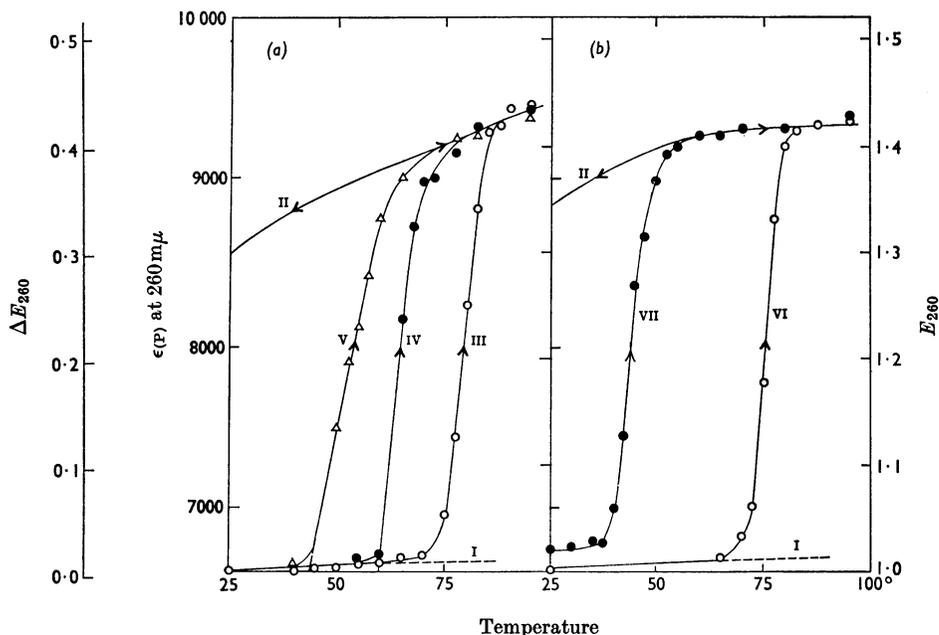


Fig. 2. Melting profiles of rat liver DNA in sodium phosphate buffer, in urea-sodium phosphate buffer and in guanidinium chloride-sodium phosphate buffer. (a) Curve I refers to the intact double-helical form, curve II is the profile for the entirely single-stranded form taken from Fig. 1(a), and curves III (○), IV (●) and V (△) respectively show the transitions from double-helical to single-stranded forms in 0.1M-, 10mM- and 1mM-sodium phosphate buffer, pH 7.6. (b) Curve I refers to the double-helical form, curve II is the profile of the denatured form in 8M-urea-10mM-sodium phosphate buffer, pH 7.6 (see Fig. 4), and curves VI (○) and VII (●) respectively show the transition from the native to denatured forms in 4M-guanidinium chloride-10mM-sodium phosphate buffer, pH 7.6, and in 8M-urea-10mM-sodium phosphate buffer, pH 7.6.

pH 7.6, was prepared. A sample was diluted with 1mM-sodium phosphate buffer, pH 7.6, to give a concentration of about 35 μg . of DNA/ml. and the u.v. spectrum was recorded. Samples of the solution were placed in the sample cell and reference cell of the recording spectrophotometer. The sample cell was then heated and the difference in the spectra of the heated sample and the sample kept at 25° was recorded at various temperatures (see Fig. 2). After the sample cell had attained 95° it was first cooled quickly in a refrigerator and then returned to the spectrophotometer. The difference in the spectra of the native and thermally denatured DNA was then recorded during a second heating cycle. The changes in the spectrum of denatured DNA with temperature are given in Fig. 1. It was noted that on heating from 25° to 95° ϵ_{260} increased gradually from about 8600 to about 9400 and ϵ_{280} from about 4600 to about 5200. The changes in extinction were reversible.

The experiment was repeated with 10mM-sodium phosphate buffer, pH 7.6, as the solvent. The extinction of denatured DNA was the same, within

experimental error, as in 1mM-sodium phosphate buffer, pH 7.6 (see Fig. 1), and the same increments in extinction were observed on raising the temperature from 25° to 95°.

The same procedure was followed when 1% formaldehyde-0.1M-sodium phosphate buffer, pH 7.2, was the solvent. The solution in the sample cell was heated to 95°, cooled and heated for a second and a third time. The changes in extinction observed on the second and successive cycles were reversible and at 260 $m\mu$ amounted to 12% of the extinction of native DNA at 25°. The reversible increments in ϵ_{260} and ϵ_{280} were close to the values noted for denatured DNA in 1mM- and 10mM-sodium phosphate buffer, pH 7.6 (Fig. 1 and Table 1).

Influence of the sodium phosphate buffer concentration on the denaturation of DNA. The profiles of ϵ_{260} against temperature are given in Fig. 2(a) for double-helical rat liver DNA in 1mM-, 10mM- and 0.1M-sodium phosphate buffer, pH 7.6. When 0.1M-sodium phosphate buffer, pH 7.6, was the solvent ϵ_{260} increased gradually by 2% when the temperature was raised from 25° to 70° (curve I in Fig. 2a)

Table 1. *Hypochromism (at 260m μ) of single-stranded polynucleotides when base-pairing is prevented*

The RNA was isolated from the larger sub-particles of reticulocyte ribosomes. The DNA ($S_{20,w}$ 21.6s) was isolated from rat liver and denatured thermally.

Solvent	RNA		DNA
	$S_{25,w}$ (s)	$1 - \epsilon_{(25)}/\epsilon_{(95)}$	$1 - \epsilon_{(25)}/\epsilon_{(95)}$
1mM-Sodium phosphate buffer, pH7.6	—	—	0.09
10mM-Sodium phosphate buffer, pH7.6	1.6*	0.10	0.09
	1.8†	0.10	—
	0.8†	0.08	—
1% Formaldehyde-0.1M-sodium phosphate buffer, pH7.6	1.8†	0.08	0.065
1% Formaldehyde-0.1M-sodium phosphate buffer, pH7.6	30‡	0.115‡	—

* Fragment obtained by hydrolysis with 0.4N-KOH at 25° for 45 min. followed by chromatography on Sephadex G-100.

† Fragment obtained by digestion with ribonuclease T₁ followed by fractionation by polyacrylamide-gel electrophoresis (see Gould, 1967).

‡ Data of Cox & Kanagalingam (1967b).

This small increase may be due to a small amount of either RNA or denatured DNA, which might be present as impurity. It is nevertheless apparent that the extinction, ϵ_{dh} , of the base residues in the double-helical form is scarcely affected by temperature. Curve I in Fig. 2(a) measures ϵ_{dh} as a function of temperature. After denaturation to a single-stranded 'stacked' form curve II in Fig. 2(a) (cf. Fig. 1) is followed. When the sodium phosphate buffer concentration was lowered more and more of curve II (Fig. 2a) was revealed. The mid-point, T_m , of the transition from curve I to curve II (Fig. 2a) calculated from both ϵ_{260} and ϵ_{280} was $80 \pm 0.5^\circ$. T_m was calculated in the same way from ϵ_{260} and ϵ_{280} when 1mM- or 10mM-sodium phosphate buffer, pH7.6, was the solvent. The value of T_m found for 10mM-sodium phosphate buffer, pH7.6, namely $64.5 \pm 0.5^\circ$, is identical with the value given by Marmur & Doty (1962) for mammalian DNA. The T_m value $51.5 \pm 0.5^\circ$ was noted when the solvent was 1mM-sodium phosphate buffer, pH7.6. In 0.1M-sodium phosphate buffer, pH7.6, the entire transition was completed on heating from 70° to 80°, and $T_m - 10^\circ$ and $T_m + 10^\circ$ were taken as convenient points of reference. The increment $\epsilon_{(T_m+10)} - \epsilon_{(T_m-10)} = \Delta\epsilon_{(m)}$ should reflect principally the transition from double-helical to denatured forms. As T_m increases so does $\epsilon_{(T_m+10)}$ whereas $\epsilon_{(T_m-10)}$ is effectively constant, so that

Table 2. *Comparison of the effects of denaturation in different solvents on the spectrum of rat liver DNA*

Solvent	$\epsilon_{280}/\epsilon_{260}$		T_m		$\epsilon_{(T_m+10)}/\epsilon_{(T_m-10)}$		$\Delta\epsilon_{280(m)}/\Delta\epsilon_{260(m)}$		$\epsilon_{(95)}/\epsilon_{(T_m-10)}$		Second heating cycle ($\Delta\epsilon_{280}/\Delta\epsilon_{260}$)d-DNA
	25°	95°	260m μ	280m μ	260m μ	280m μ	260m μ	280m μ	260m μ	280m μ	
1mM-Sodium phosphate buffer, pH7.6	0.546	0.557	51.5°	52°	1.365	1.334	0.574	0.574	1.43	1.45	0.64
10mM-Sodium phosphate buffer, pH7.6	0.552	0.546	64.5	65.5	1.39	1.38	0.550	0.550	1.42	1.44	0.71
0.1M-Sodium phosphate buffer, pH7.6	0.543	0.585	79	79.5	1.415	1.43	0.555	0.555	1.42	1.44	0.55
8M-Urea-10mM-sodium phosphate buffer, pH7.6	0.570	0.593	44	44.5	1.38	1.41	0.610	0.610	1.40	1.46	0.65
4M-Guanidinium chloride-10mM-sodium phosphate buffer, pH7.6	0.548	0.580	75.5	75.5	1.41	1.47	0.645	0.645	1.41	1.49	0.65
1% Formaldehyde-0.1M-sodium phosphate buffer, pH7.6	0.543	0.663	68	68	1.49	1.73	0.834	0.834	1.52	1.78	0.8

$\Delta\epsilon_{260(m)}$ and $\Delta\epsilon_{280(m)}$ are the increments at 260 and 280m μ respectively observed on raising the temperature from $T_m - 10^\circ$ to $T_m + 10^\circ$. $\Delta\epsilon_{260}$ and $\Delta\epsilon_{280}$ are the increments in ϵ at 260 and 280m μ respectively observed on raising the temperature from 25° to 95° of solutions of DNA previously denatured by heating to 95°.

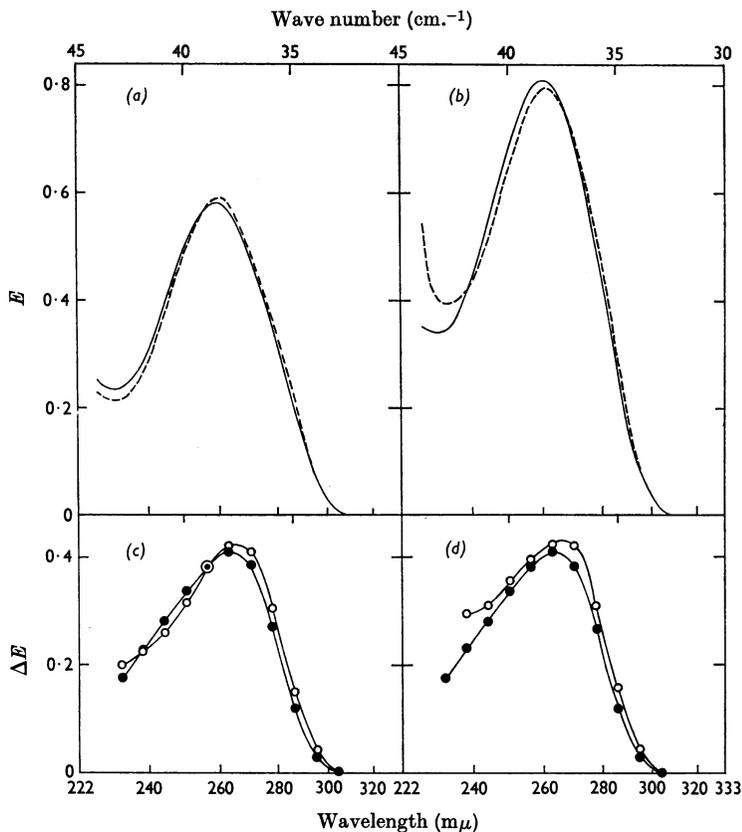


Fig. 3. Spectrum of rat liver DNA in sodium phosphate buffer and in guanidinium chloride-sodium phosphate buffer. (a) Spectrum of native DNA at 25°: —, 10mM- or 0.1M-sodium phosphate buffer, pH 7.6; ----, 4M-guanidinium chloride-10mM-sodium phosphate buffer, pH 7.6; E_{260} was found to be greater by 0.01 when 8M-urea-10mM-sodium phosphate buffer, pH 7.6, was the solvent, but otherwise the spectrum was the same as in 4M-guanidinium chloride-10mM-sodium phosphate buffer, pH 7.6. (b) Spectrum as for (a) but at 95°; the values of extinction were not corrected for a decrease of 3.1% in the concentration of DNA due to the thermal expansion of water. (c) Comparison of the difference spectra $E_{(T_m+10)} - E_{(T_m-10)}$ of rat liver DNA in 0.1M-sodium phosphate buffer, pH 7.6 (●), and in 4M-guanidinium chloride-10mM-sodium phosphate buffer, pH 7.6 (○). (d) Comparison of the difference spectra $E_{(T_m+10)} - E_{(T_m-10)}$ of rat liver DNA in 0.1M-sodium phosphate buffer, pH 7.6 (●) (as for c), and $E_{(87.5)} - E_{(25)}$ of rat liver DNA in 8M-urea-10mM-sodium phosphate buffer, pH 7.6 (○).

$\Delta\epsilon_{(T_m+10)}/\Delta\epsilon_{(T_m-10)}$ should increase with T_m . This was found to be the case (see Table 2). The ratio $\Delta\epsilon_{280(m)}/\Delta\epsilon_{260(m)}$, which has been interpreted to yield the nucleotide composition of DNA (Felsenfeld & Sandeen, 1962; Fresco *et al.* 1963), is given in Table 2. At 95° the extinctions, $\epsilon_{(95)}$, were similar if not identical in each solvent, so that $\epsilon_{(95)}/\epsilon_{(T_m-10)}$ should also be independent of the buffer concentration: this was observed (see Table 2). The results show that eqn. (1) is valid when T_m is close to 95°.

Denaturation in the presence of urea or guanidinium chloride. The spectra of rat liver DNA in 8M-urea-10mM-sodium phosphate buffer, pH 7.6, and in 10mM-sodium phosphate buffer, pH 7.6, were similar but not identical (Fig. 3a); ϵ_{260} was increased

by 1.5%, $\epsilon_{280}/\epsilon_{260}$ was slightly increased (see Table 2) and the maximum was displaced from 259 to 261 m μ when urea was present. Raising the temperature led to denaturation at T_m 44° (Fig. 2b) compared with 64.5 (Fig. 2a) when urea was omitted. The ratio $\epsilon_{260(95)}/\epsilon_{260(T_m-10)}$ was almost the same in the presence as in the absence of 8M-urea whereas $\epsilon_{280(95)}/\epsilon_{280(T_m-10)}$ was about 10% greater when 8M-urea was present. The difference in the spectra of the native and denatured forms, $E_{(T_m+10)}/E_{(T_m-10)}$, was measured over the range 230–310 m μ both when 8M-urea was present and when urea was absent (Fig. 3d). When urea was present $E_{(87.5)} - E_{(25)}$ was the same as $E_{(T_m+10)} - E_{(T_m-10)}$ to within $\pm 5\%$. High precision in

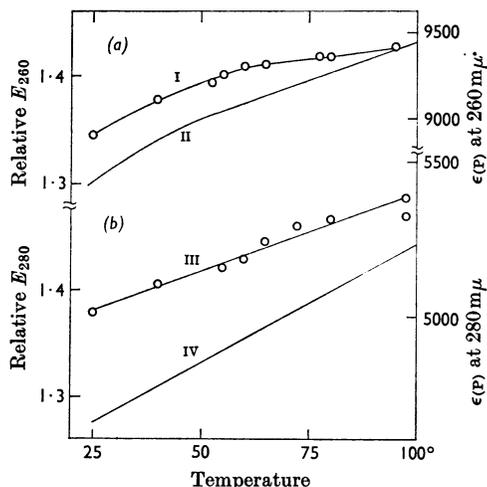


Fig. 4. Effect of urea on the hypochromism of denatured DNA in sodium phosphate buffer. Curves I and III, solvent 8M-urea-10mM-sodium phosphate buffer, pH 7.6; curves II and IV, solvent 1mM- or 10mM-sodium phosphate buffer, pH 7.6 (see Fig. 1). The extinctions at 260 and 280 $m\mu$ are relative to those noted for native DNA at 25°.

Table 3. Effect of sodium chloride concentration on the reduced specific viscosity of denatured calf thymus DNA

Concn. of NaCl (mM)	$\eta_{sp.}/c$ (ml./g.)	
	25°	55°
0.125	6100	6800
3.1	1700	1800
6.1	1040	1300
12.0	810	900

values of E at wavelengths shorter than 230 $m\mu$ is difficult to attain because of the high extinction of urea. The presence of 8M-urea led to a slight shift in the difference spectrum to longer wavelengths, e.g. ϵ_{max} was found at 263 $m\mu$ (ν 38 cm^{-1}) in 0.1M-sodium phosphate buffer, pH 7.6, and at 267 $m\mu$ (ν 37.5 cm^{-1}) in 8M-urea-10mM-sodium phosphate buffer, pH 7.6; the values of $\Delta\epsilon_{280(m)}/\Delta\epsilon_{260(m)}$ were respectively 0.555 and 0.610 (see Table 2). Thus the effect of urea on the denaturation spectrum of DNA is small, especially at 260 $m\mu$. The spectrum of DNA at 95° is slightly affected by urea (Fig. 3b) since $\epsilon_{280}/\epsilon_{260}$ at 95° may be slightly greater when urea is present (cf. Stockx, 1963).

The profile of ϵ_{260} against temperature was found to be slightly sharper when urea was present in the solvent. Curve II in Fig. 2(b) was obtained by heating DNA denatured in 8M-urea-10mM-sodium phosphate buffer, pH 7.6, and its slope was less than that of curve II in Fig. 2(a), which was obtained when urea was absent. The difference in

the slope of curve II in Fig. 2(a) and curve II in Fig. 2(b) is reflected in the increment $\epsilon_{260}(T_m+10) - \epsilon_{260}(T_m-10)$. In the absence of urea this difference, $\Delta\epsilon_{260(m)}$, was found to increase as T_m increased (Fig. 2a). However, $\Delta\epsilon_{260(m)}$ was 38% (T_m 44°) in 8M-urea-10mM-sodium phosphate buffer, pH 7.6, compared with 36.5% (T_m 51.5°) in 1mM-sodium phosphate buffer, pH 7.6.

The spectrum of native DNA appeared to be the same in 4M-guanidinium chloride-10mM-sodium phosphate buffer, pH 7.6, as in 0.1M-sodium phosphate buffer, pH 7.6, e.g. ϵ_{260} and $\epsilon_{280}/\epsilon_{260}$ (Fig. 3d) were scarcely affected. When the solution was heated denaturation was found to take place at 75.5° in 4M-guanidinium chloride-10mM-sodium phosphate buffer, pH 7.6, compared with 79° in 0.1M-sodium phosphate buffer, pH 7.6. The increment $\epsilon_{260}(T_m+10) - \epsilon_{260}(T_m-10)$ was 5% smaller whereas $\epsilon_{280}(T_m+10) - \epsilon_{280}(T_m-10)$ was 15% greater in 4M-guanidinium chloride-10mM-sodium phosphate buffer, pH 7.6, than in 0.1M-sodium phosphate buffer, pH 7.6. The difference spectrum $E_{(95)} - E_{(T_m-10)}$ was identical within experimental error with that observed when 8M-urea-10mM-sodium phosphate buffer, pH 7.6, was the solvent (see Table 2 and Fig. 3).

Effect of urea on the hypochromism of single-stranded polynucleotides. The dependence on temperature of ϵ_{260} and ϵ_{280} of denatured DNA in 10mM-sodium phosphate buffer, pH 7.6, and in 8M-urea-10mM-sodium phosphate buffer, pH 7.6, are compared in Fig. 4. The hypochromism observed is decreased to about 5% by the addition of urea and $\Delta\epsilon_{280}/\Delta\epsilon_{260}$ (Table 2) decreased from 0.71 to 0.65.

Influence of sodium chloride concentration and temperature on the viscosity of denatured DNA. Calf thymus DNA (2mg.) was dissolved in twice-glass-distilled water (10ml.) and denatured by the addition of hydrochloric acid at 25° to a final concentration of 1mM. The solution was neutralized with potassium hydroxide. A sample was diluted to 50 $\mu g./ml.$ and the viscosity was measured at 25° and at 55° as described by Littauer & Eisenberg (1959) and Cox & Littauer (1962). The concentration of sodium chloride was increased by the addition of 2M-sodium chloride delivered from an Agla micrometer syringe. The viscosity was measured at 25° and 55° for a range of sodium chloride concentrations. Increasing the DNA concentration to 100 $\mu g./ml.$ or decreasing it to 25 $\mu g./ml.$ did not alter appreciably the observed values of $\eta_{sp.}/c$. Representative results are given in Table 3, which shows that increasing the temperature from 25° to 55° leads to an increase of about 10% in $\eta_{sp.}/c$. However, a tenfold increase in the concentration of sodium chloride led to a threefold decrease in viscosity.

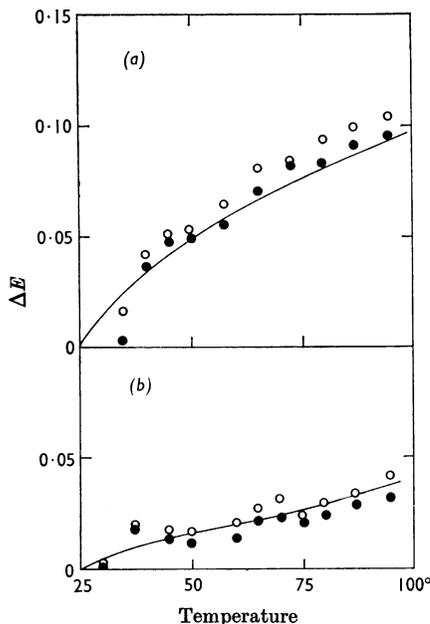


Fig. 5. Hypochromism of fragments of RNA isolated from the larger sub-particle of rabbit reticulocyte ribosomes. RNA was degraded either with alkali (Cox, Gould & Kanagalingam, 1968) or with ribonuclease T_1 (Gould, 1967). The fragments were fractionated according to size either by chromatography on Sephadex G-100 or by polyacrylamide-gel electrophoresis. Fragments of $S_{20,w}$ 0.8–1.8s had essentially the same values as those given in (a) and (b) for 1.8s fragments. (a) Solvent 0.1 M-sodium phosphate buffer, pH 7.6. (b) Solvent 4 M-guanidinium chloride–10 mM-sodium phosphate buffer, pH 7.6. The full line in (a) was observed for both ϵ_{260} and ϵ_{280} on heating the fragments in 1% formaldehyde–0.1 M-sodium phosphate buffer, pH 7.6, for the second and successive heating cycles. The values of ΔE refer to a solution of E_{260} 1.0 at 25°. \circ , E_{260} ; \bullet , E_{280} .

Hypochromism of entirely single-stranded RNA. RNA from the larger sub-particle of rabbit reticulocyte ribosomes was hydrolysed either by alkali or by enzyme until the weight-average molecular weight was about 15000 and about 80% of the double-helical structure was destroyed. The fragments were then fractionated according to size either by chromatography on Sephadex G-100 (cf. Spencer & Poole, 1965) or by polyacrylamide-gel electrophoresis (Gould, 1967) to isolate fragments that had fewer than the minimum residues necessary to form a hairpin loop (for theoretical analysis see Cox, 1968). The denaturation spectrum of fractions of $S_{20,w}$ 0.8–1.8s did not depend appreciably on size, in accord with the view that these fragments are too small to form intramolecular double-helical secondary structure in 10 mM-sodium phosphate buffer, pH 7.6, at 25°. A sample of the

unfractionated hydrolysate was treated with formaldehyde and the reversible hypochromism is given as the continuous line in Fig. 5(a) together with the observed hypochromism of a fraction of fragments of 1.8s. When 4 M-guanidinium chloride–10 mM-sodium phosphate buffer, pH 7.6, was the solvent the hypochromism was decreased to about 4% (Fig. 5b), which is comparable with the effect of 4 M-guanidinium chloride on the hypochromism of oligoadenylic acid and oligoguanylic acid (Cox & Kanagalingam, 1967b) and is to be compared with a decrease to about 5% noted for denatured DNA. When the intact (30s) molecule was treated with formaldehyde to prevent base-pairing an increase of 14% in ϵ_{260} or ϵ_{280} was found on heating from 25° to 95°, an increment that is about the same as that found for rat liver DNA (Table 1).

DISCUSSION

Hypochromism of entirely single-stranded polynucleotides. (a) Denatured DNA in 1 mM- and 10 mM-sodium phosphate buffer, pH 7.6. The available evidence suggests (e.g. Cox & Kanagalingam, 1967a,b) that hypochromism due to 'stacking' in single-stranded regions is not as dependent on the electrolyte concentration as the hypochromism due to double-helical structures. For native DNA, within the limits of 1 mM- and 1 M-sodium chloride, the temperature, T_m , at which 50% of DNA becomes denatured varies with the molar sodium chloride concentration, C_{salt} , according to the equation:

$$T_m = 16.6 \log C_{\text{salt}} + 102 \quad (2)$$

i.e. T_m increases by about 16.6° when the electrolyte concentration is increased tenfold (Schildkraut & Lifson, 1965), as shown in Fig. 2. It was found (Fig. 1) that after rat liver DNA was heated to 95° in 1 mM- or 10 mM-sodium phosphate buffer, pH 7.6, the reversible dependence of extinction on temperature was the same in both solvents. This is a strong indication that double-helical secondary structure is absent from denatured rat liver DNA at 25° or above in these solvents.

(b) Denatured DNA after reaction with formaldehyde. Stollar & Grossman (1962) showed that if DNA isolated from bacteriophage T_4 is treated in 1% formaldehyde the single strands do not recombine to form a double helix, though recombination takes place readily in the absence of formaldehyde. The single-stranded character of DNA after reaction with formaldehyde was confirmed by measurement of the mass per unit length (Luzzati, Mathis, Masson & Witz, 1964). In contrast, the optical properties of single-stranded polynucleotides that are devoid of double-helical character in neutral salt solutions remain largely unchanged after

reaction with formaldehyde (Fasman, Lindblow & Grossman, 1964; Stevens & Rosenfeld, 1966). When RNA was heated to 95° in 1% formaldehyde the formation of even very short double-helical segments was prevented. It was found that the reversible hypochromism of RNA in 1% formaldehyde was scarcely dependent on chain length over the range 2.7–30s whereas the hypochromism found in the absence of formaldehyde was profoundly affected by degradation from 30s to 2.7s RNA (Cox *et al.* 1968). Hypochromism due to 'stacking' between unpaired residues is known to be only slightly dependent on chain length (e.g. Leng & Felsenfeld, 1966). Thus heating at 95° in 1% formaldehyde appears to be sufficient to prevent base-pairing. Higher concentrations of formaldehyde were avoided, because it is also a denaturant that affects the T_m of native DNA and the possibility exists that the extent of 'stacking' between unpaired residues might also be affected.

It is inferred that both procedures measure hypochromism due to 'stacking' in single-stranded regions. Though reaction with formaldehyde may affect the hypochromism of 'stacked' unpaired residues the effect is not large, since the hypochromism observed after reaction with 1% formaldehyde is not less than 80% of the value found at low ionic strengths in the absence of formaldehyde (see Table 1). The hypochromism of polyadenylic acid decreased by about 20% after reaction with 2% formaldehyde (Stevens & Rosenfeld, 1966). The notion that the reaction of a polynucleotide with formaldehyde prevents base-pairing and yields an approximate value for the hypochromism of the single strand appears justified by the results given in Fig. 1.

The data of Fig. 1 are also in accord with the hypochromism of denatured DNA in 10mM-phosphate buffer reported by Eigner & Doty (1965) and with the reversible hypochromism of DNA from bacteriophage T₄ in formaldehyde solutions (Stollar & Grossman, 1962).

The extinction coefficient ϵ_s is given by the equation:

$$\epsilon_s = f_s \epsilon_s^0 + (1 - f_s) \epsilon_{am}^0 \quad (3)$$

where f_s is the fraction of residues that are 'stacked' and ϵ_s^0 and ϵ_{am}^0 are average extinctions per nucleotide when the polynucleotide is in the entirely 'stacked' or completely amorphous conformation respectively, so that the fraction of residues that are 'stacked' is:

$$f_s = (\epsilon_{am}^0 - \epsilon_s) / (\epsilon_{am}^0 - \epsilon_s^0) \quad (4)$$

It is likely that when all the residues are 'stacked' the extinction per base residue is the same as in the double helix, i.e. $\epsilon_s^0 = \epsilon_{dh}^0$ where ϵ_{dh}^0 is the value of $\epsilon_{(P)}$ (6600) for native DNA. It appears that f_s

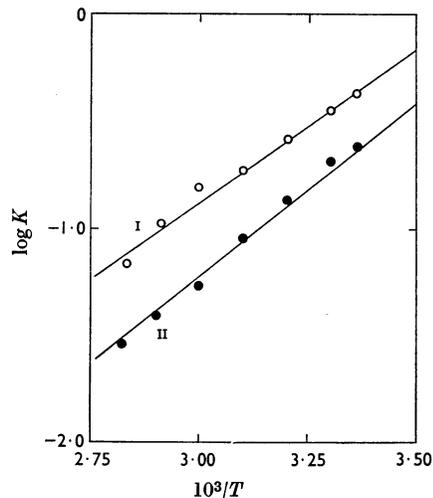


Fig. 6. Van't Hoff plots of the 'melting' data (Fig. 4) of denatured DNA in 1mM- or 10mM-sodium phosphate buffer, pH 7.6 (curve I), and in 8M-urea-10mM-sodium phosphate buffer, pH 7.6 (curve II). K is defined by eqn. (5).

calculated by means of eqn. (4) is about 0.30. If $\epsilon_{am}^0 - \epsilon_s^0$ is less than $\epsilon_{am}^0 - \epsilon_{dh}^0$ then f_s will be greater than 0.30, which is likely to be a minimum value. Where urea was absent f_s was calculated to be 0.30 and 0.32 from ϵ_{260} and ϵ_{280} respectively, compared with 0.18 and 0.20 respectively when urea was present. Apparently urea decreased 'stacking' among the unpaired residues of rat liver DNA by about 40%.

The constant, K , for the equilibrium between the amorphous and 'stacked' forms is the same as for an equivalent mixture of dinucleotides (Brahms, Michelson & Van Holde, 1966), since the reaction is not co-operative. Hence:

$$K = f_s / (1 - f_s) = (\epsilon_{am}^0 - \epsilon_s) / (\epsilon_s - \epsilon_{dh}^0) \quad (5)$$

on the basis of the assumption that $\epsilon_s^0 = \epsilon_{dh}^0$. The extinction at 95° is assumed to be ϵ_{am}^0 . Thus K may be evaluated at different temperatures and since $\partial \ln K / \partial (1/T) = \Delta H^0 / R$ the standard-state enthalpy, ΔH^0 and entropy, ΔS^0 , may be calculated. Values of K were calculated from the data of Fig. 1 and a plot of $\log K$ against $1/T$ yielded a straight line (Fig. 6). Values of ΔH^0 and ΔS^0 were calculated to be 6.5 kcal./mole and 17 e.u./mole respectively. At 25° the free energy is about 0.5 kcal./mole in favour of the amorphous form. It is estimated that the uncertainty in either ϵ_{am}^0 or ϵ_s^0 could at most lead to a $\pm 10\%$ error in the values of enthalpy and entropy, so that the overall limits in ΔH^0 and ΔS^0 should not be greater than $\pm 20\%$.

The calculated values of enthalpy and entropy are to be compared with the values of 8 kcal./mole

and 28 e.u./mole respectively obtained at pH 7 for polyadenylic acid (Brahms *et al.* 1966), which is known to have a single-stranded 'stacked' structure.

The values of ΔH^0 and ΔS^0 were calculated to be 7.4 kcal./mole and 20 e.u./mole respectively for denatured DNA in 8M-urea-10mM-sodium phosphate buffer, pH 7.6, from the data of Fig. 4; the free energy in favour of the amorphous form was estimated as 0.8 kcal./mole at 25°.

Effects of urea and guanidinium chloride on the spectrum of DNA. Both 8M-urea and 4M-guanidinium chloride perturbed the spectrum of DNA at 95° whereas the spectrum of native DNA at 25° was scarcely affected (Fig. 3). Consequently there is a small difference in the denaturation spectrum of DNA depending on the solvent (see Fig. 3c). The perturbation of the spectrum of denatured DNA, but not that of native DNA, is in accord with the view (Hanlon, 1966) that denaturants act as an effective competitor for the DNA base residues.

The T_m of DNA in 0.1M-sodium phosphate buffer, pH 7.6, was 79° compared with 75.5° in 4M-guanidinium chloride-10mM-sodium phosphate buffer, pH 7.6. This difference of 3.5° is in accord with the view that T_m is decreased by about 20° by the hydrogen-bond-breaking properties of the guanidinium ion (cf. 8M-urea), but that this is offset by about 16° (see eqn. 2) because the electrolyte concentration is increased from 0.1M to 1M or more (increasing the salt concentration beyond 1M has no further effect on T_m). The T_m of the triple-stranded complex poly(A+2U) formed between one strand of polyadenylic acid and two strands of polyuridylic acid is 80° in 1M-sodium chloride but is 56° in 8M-urea-1M-potassium chloride, and is 96° in 4M-sodium chloride (value obtained by extrapolation from the data of Stevens & Felsenfeld, 1964), but is 73° in 4M-guanidinium chloride-10mM-sodium phosphate buffer, pH 7.6. The difference in T_m of DNA in 8M-urea-10mM-sodium phosphate buffer, pH 7.6, and in 4M-guanidinium chloride-10mM-sodium phosphate buffer, pH 7.6, is 31°, which is also attributable to the difference in the ionic strength of the two solvents. Thus the denaturation of DNA in 4M-guanidinium chloride-10mM-sodium phosphate buffer, pH 7.6, appears to be equivalent to denaturation in 8M-urea-10mM-sodium phosphate buffer, pH 7.6.

Both 8M-urea and 4M-guanidinium chloride decrease hypochromism due to 'stacking' to about 30-40% (see Fig. 4). Consequently it was found that the transition from the native to denatured forms was found to be sharper in 8M-urea-10mM-sodium phosphate buffer, pH 7.6, than in 1mM-sodium phosphate buffer, pH 7.6, even though T_m was about the same (Fig. 2).

Comparison of the hypochromism of the entirely single-stranded forms of RNA and DNA. Two

methods of obtaining single-stranded polynucleotides devoid of double-helical secondary structure were discussed above: the first was chemical modification of the base residues by reaction with formaldehyde and the second method was to decrease the ionic strength. A third method has been applied to ribosomal RNA. Hypochromism due to 'stacking' arises from a non-co-operative interaction between adjacent base residues and so is scarcely dependent on the chain length of the polynucleotides (e.g. Leng & Felsenfeld, 1966). The fraction of base residues 'stacked' but unpaired in the intact molecule when base-pairing is prevented is defined as f_s^0 , and random degradation to a fragment having a number-average chain length of $1/p$ residues, where p is the probability of a diesterified phosphate linkage being hydrolysed, will decrease the fraction of 'stacked' residues to f_s , where:

$$f_s = f_s^0(1-p)^2 \quad (6)$$

(see Cox *et al.* 1968).

In contrast, the formation of double-helical secondary structure involves a larger sequence of 9 or more nucleotides. Suppose that a hairpin loop is formed from a sequence of i residues and that there are l^0 loops in the intact molecule and l intact loops are formed by the fragments. The probability that the i residues of a loop will remain united after hydrolysis is $(1-p)^i$, which is also equal to the fraction of loops remaining intact, i.e.:

$$l = l^0(1-p)^i \quad (7)$$

Since i is not likely to be much less than about 20 residues, the capacity of a polynucleotide to form double-helical secondary structure will diminish far faster on degradation than the capacity of adjacent unpaired residues to 'stack' one upon another. Degradation to fragments of 1.8s or less is sufficient to prevent the formation of double-helical structure.

Though the hypochromism due to 'stacking' among unpaired residues is about the same for DNA and RNA it appears that the optical rotatory dispersion is different for polyribonucleotides and polydeoxyribonucleotides (Ts'o, Rapaport & Bollum, 1966). It appears that the presence or absence of the C₍₂₎-hydroxyl group affects optical rotatory dispersion to a far greater extent than hypochromism.

Conformation of denatured DNA in 10mM-sodium phosphate buffer, pH 7.6. The evidence presented above suggests that base-paired regions are absent from denatured rat liver DNA in 10mM-sodium phosphate buffer, pH 7.6, at 25°. The hydrodynamic properties of denatured DNA in 10mM-sodium phosphate buffer, pH 7.6, support this view. The reduced specific viscosity η_{sp}/c of calf thymus DNA

was measured at different concentrations of sodium chloride (Table 3). The high viscosity observed over the range 0.5–10mm-sodium chloride shows that the form of the polynucleotide that is present in very low salt concentrations persists in 10mm-sodium chloride solutions. When the sodium chloride concentration is increased above about 0.5 mm the viscosity of a flexible high-molecular-weight polyelectrolyte will decrease according to the relation:

$$[\eta] = [\eta_0](C_{\text{salt}}/C_{\text{salt}}^0)^{-0.6} \quad (8)$$

when $[\eta]$ and $[\eta_0]$ are the limiting viscosity numbers ($[\eta] = \eta_{\text{sp}}/c$ when $c \rightarrow 0$) in solutions of electrolyte of concentrations of C_{salt} and C_{salt}^0 respectively (Cox, 1960). C_{salt}^0 is any convenient reference solution.

Denatured mammalian DNA behaves as a simple polyelectrolyte over the range 0.5–10mm-sodium chloride, i.e. the viscosity decreased to about one-third when the sodium chloride concentration was increased tenfold (Table 3). The values of viscosity obtained at 25° and 55° differed by no more than about 10%. Polyuridylic acid is known to have little or no organized secondary structure at 25° and its viscosity also varies with the concentration of electrolyte to an extent that is entirely attributable to the polyelectrolyte nature of the sugar phosphate backbone (Richards, Flessel & Fresco, 1963). The behaviour of denatured mammalian DNA differs in two respects from the behaviour of RNA (Cox & Littauer, 1962), which is known to form double-helical secondary structure in 10mm-sodium chloride solutions: (a) the viscosity of *Escherichia coli* ribosomal RNA decreases about 12-fold over the range 1–10mm-sodium chloride and this anomalous contraction is concomitant with the formation of double-helical secondary structure (Cox & Littauer, 1962); (b) the viscosity was found to depend on temperature over the range 25–55° owing to changes in the extent of double-helical secondary structure (for review see Spirin, 1963).

Further, the relation between $[\eta]$ and molecular weight, M , and between the sedimentation coefficient, S , and M is also informative about the shape of the polynucleotide in solution. In standard saline-citrate solution the relations found for polyuridylic acid (Richards *et al.* 1963), which has no organized secondary structure, are:

$$S_{20,w} = 3.29 \times 10^{-2} M^{0.42} \quad (9)$$

and, at 25°:

$$[\eta] = 8.98 \times 10^{-5} M^{0.75} \quad (10)$$

The corresponding relations found by Eigner & Doty (1965) for denatured DNA of 50% G+C content in 10mm-phosphate buffer are:

$$S_{25,w} = 5.6 \times 10^{-2} M^{0.36} \quad (11)$$

and:

$$[\eta] = 3.1 \times 10^{-4} M^{0.91} \quad (12)$$

from which it is inferred that in 10mm-sodium phosphate buffer at 25° denatured DNA has a hydrodynamic volume consistent with the absence of organized secondary structure.

In contrast, the relations (Hall & Doty, 1958) for calf liver ribosomal RNA in 10mm-phosphate buffer at 25° are:

$$S_{25}^0 = 2.1 \times 10^{-2} M^{0.49} \quad (13)$$

and:

$$[\eta] = 6.2 \times 10^{-4} M^{0.53} \quad (14)$$

which shows that RNA forms an unusually compact coil in this solvent at 25°, probably owing to intramolecular double-helical structure. That the RNA chain occupies an unusually small hydrodynamic volume is implied by the increased dependence of S and the decreased dependence of $[\eta]$ on M : for less compact conformations an exponent smaller than 0.49 in eqn. (13) and an exponent greater than 0.53 in eqn. (14) would be predicted. Thus the hydrodynamic data (eqns. 8–14 and Table 3) show that the conformations of calf liver ribosomal RNA and denatured DNA at 25° in 10mm-sodium phosphate buffer differ significantly. Both the hypochromic and the hydrodynamic properties of denatured DNA in 10mm-sodium phosphate buffer at 25° are consistent with the absence of double-helical secondary structure.

Conformation of denatured DNA in 0.1M-sodium phosphate buffer, pH 7.6. Whereas increasing the concentration of phosphate buffer from 1mm to 10mm did not affect the hypochromism of denatured DNA (see Fig. 1), increasing the buffer concentration to 0.1M led to a decrease in the extinction at 25° from 1.30 to 1.18 (cf. 1.00 for native DNA). When the solution was heated the extinction increased gradually until at about 60° and above the same values were found as in 10mm-sodium phosphate buffer, pH 7.6 (see Fig. 1). The extinction of denatured DNA was about the same over the range 25–95° in 4M-guanidinium chloride–10mm-sodium phosphate buffer, pH 7.6, as in 0.1M-sodium phosphate buffer, pH 7.6 (Fig. 7).

It is inferred that double-helical secondary structure is formed at 25° when the buffer concentration is increased to 0.1M. The fraction, f_{dh} , of residues that are base-paired is given by the equation (Boedtker, 1967; Cox & Kanagalingam, 1967a,b):

$$f_{\text{dh}} = (\epsilon_s - \epsilon) / (\epsilon_s - \epsilon_{\text{dh}}^0) \quad (15)$$

where ϵ is the observed extinction coefficient, provided that the contribution of base-paired residues to hypochromism is independent of the contribution of unpaired residues. Values of ϵ_s

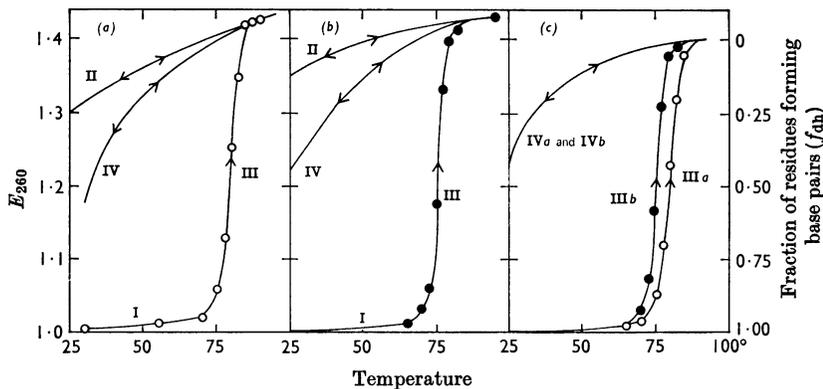


Fig. 7. Melting profile of denatured rat liver DNA in (a) 0.1 M-sodium phosphate buffer, pH 7.6, and in (b) 4 M-guanidinium chloride-10 mM-sodium phosphate buffer, pH 7.6. Curve I refers to the double-helical form, curve II to the denatured entirely single-stranded form (see Figs. 1a and 4a), curve III shows the observed transition from curve I to curve II, and curve IV is the profile observed for denatured DNA in the above-mentioned solvents. (c) Plot of the fraction, f_{dh} , of residues forming base pairs calculated by means of eqn. (15). The values of f_{dh} calculated for curve IV in (a) and for curve IV in (b) were similar (curve IVa and IVb in c).

were equated with the values of ϵ given in Figs. 1 and 4. ϵ_{dh}^0 was assumed to be the same as for native DNA, though it is possible that a somewhat higher value may apply to very short double-helical segments. Hence the calculated values of f_{dh} are likely to be minimum values. In Fig. 7(c) f_{dh} is given as a function of temperature for DNA in 0.1 M-sodium phosphate buffer, pH 7.6, and in 4 M-guanidinium chloride-10 mM-sodium phosphate buffer, pH 7.6. In both solvents f_{dh} was calculated to be 0.4 at 25° and to decrease to about 0.15 at 50° and 0.05 at 60°. It is known (e.g. Doty, 1961; Lipsett, Heppel & Bradley, 1961; Lipsett, 1964; Bautz & Bautz, 1964) that for very short double-helical segments T_m is dependent on the number of base pairs per double-helical region. The large difference (about 50°) in the T_m values of native and denatured states implies that double-helical segments of about 3-5 base pairs are involved in the secondary structure of denatured DNA in 0.1 M-sodium phosphate buffer.

It is not possible to decide from the hypochromism data whether the double-helical structure is formed from inter- or intra-molecular interactions. However, it was found (Eigner & Doty, 1965) that increasing the salt concentration from 10 mM-phosphate buffer to 0.15 M-sodium chloride-15 mM-sodium citrate led to an anomalous contraction of the polynucleotide chain, since there was a 12-fold decrease in the viscosity instead of the threefold change predicted by eqn. (8) for simple polyelectrolyte behaviour, and that $S_{25,w}^0$ and $[\eta]$ were related to molecular weight by the equations:

$$S_{25,w}^0 = 2.2 \times 10^{-2} M^{0.48} \quad (16)$$

and:

$$[\eta] = 4.9 \times 10^{-3} M^{0.55} \quad (17)$$

These relations show that in 0.1 M-sodium phosphate buffer denatured DNA forms a more compact coil than an amorphous polynucleotide such as polyuridylic acid (cf. eqns. 9 and 10). The anomalous contraction of the molecule and the similarity between eqns. (13) and (16) and between eqns. (14) and (17) suggest that the double-helical segments arise from intramolecular interactions as in RNA.

Conclusions. When nucleic acids are prevented from forming double-helical structures the hypochromism due to 'stacking' between adjacent unpaired residues is significant at 25° and may amount to 28% or more of the hypochromism found for the intact double helix. The magnitude of this effect is about the same for both RNA and DNA (Table 1), in contrast with optical rotatory dispersion (Ts'o *et al.* 1966), which appears to be much more strongly influenced by the presence or absence of the $C'_{(2)}$ -hydroxyl group.

It appears that urea or guanidinium chloride has only a small effect on the hypochromism of multi-helical structures, but diminishes the hypochromism of a single-stranded polynucleotide. Provided that the decrease in the T_m of the multi-helix is countered by increasing the electrolyte concentration solvents such as urea may be used to measure the difference in the spectrum due to denaturation of multi-helical structures (e.g. Cox & Kanagalingam, 1967b).

The methods available for preventing the formation of double-helical structures without destroying 'stacking' between adjacent base residues include: (1) decreasing the electrolyte concentration to eliminate double-helical structure; (2) chemically modifying the base residues to prevent the formation of hydrogen bonds between complementary base pairs, e.g. by reaction with formaldehyde; (3) decreasing the chain length to fragments that are too short to form intramolecular double-helical structures.

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