A Possible Method for Characterizing the Secondary Structure of Ribonucleic Acids

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The E_{280}/E_{260} ratio was found to be suitable for following the ionization of cytosine residues of polynucleotides on the basis of studies with model compounds such as oligoguanlylic acid, oligocytidylic acid, a complex formed between polyadenylic acid and polyuridylic acid, and a copolymer of guanylic acid and cytidylic acid, provided that changes in secondary structure were taken into account. The pK of cytosine residues of a polynucleotide in the amorphous form was found to be 4.70 at 25° in 0.1 M-sodium phosphate on the basis of titration at 75–85° and on the assumption that the heat of ionization was the same as the value (5.2 kcal./mole) found for CMP. In contrast, the pK of cytosine residues in the double-helical form of DNA was found to be about 3.25. These observations were utilized in estimating the fraction of cytosine residues in helical segments of ribosomal RNA, a copolymer of guanylic acid and cytidylic acid, and a copolymer of adenylic acid, guanylic acid, uridylic acid and cytidylic acid. The ionization of guanine and uracil residues was estimated from changes in the E_{270}/E_{260} ratio and E_{230}/E_{260} ratio respectively. In the amorphous form of RNA both residues had the same pK , whereas in the double-helical form ionization was suppressed. The fraction of guanine and uracil residues in amorphous segments may be estimated from the titration curves. The difference in the denaturation spectrum of adenine--uracil and guanine--cytosine base pairs at 280 m μ was enhanced in acidic solutions whereas E_{260} was hardly affected. Hence a comparison of the increments in E_{280} and E_{260} obtained on increasing the temperature at constant pH may be used to distinguish the melting ranges of helical domains differing in nucleotide composition. In alkaline solutions comparison of the increments in E_{260} and E_{270} yields similar information. In acidic solutions the fraction of cytosine residues involved in helical secondary structure, the degree of ionization of cytosine residues and the fraction of adenine--uracil base pairs denatured may be estimated from ΔE_{265} and ΔE_{280} . In alkaline solutions the fractions of guanine and uracil residues involved in secondary structure and the degrees of ionization of these residues may be estimated from ΔE_{230} , ΔE_{245} , ΔE_{260} and ΔE_{280} .

The general features of the secondary structure of RNA are well known (for reviews see Spirin, 1963; Cox, 1965a), in particular the tendency to form weak intramolecular helical structures. However, knowledge of the nucleotide composition, the size of helical segments and the base-pairing involved is not precise. Accordingly a more detailed study of the secondary structure of ribosomal RNA was attempted (Cox, 1966a) on the basis of the assumptions that when base residues interact to form helical structures their dissociation constants are considerably modified (Cox, 1961), that the difference in the ultraviolet-absorption spectrum can be analysed to yield the nucleotide composition of helical segments 'melting' to an amorphous form over the temperature range studied (Felsenfeld &

Sandeen, 1962; Fresco, Klotz & Richards, 1963), and that a helical segment is best characterized by the effects of pH and ionic strength on its thermal stability. The aim of the present work is to provide stricter criteria for the denaturation of A--U*

* Abbreviations: A, G, U and C, adenine, guanine, uracil and cytosine residues respectively; oligoA etc., oligoadenylic acid etc.; polyA etc., polyadenylic acid etc.; polyAU etc., copolymer of adenylic acid and uridylic acid etc.; poly(A+U) etc., complex of polyA and polyU etc.; A--U etc., base residues indicated paired in authentic double-helical structure in which the base residues are located on separate segments of either the same or different polynucleotide chains; A--aq. etc., (probably solvated) adenine residues etc. in the amorphous form of a polynucleotide; TMV, tobacco mosaic virus.

and G-C base pairs. The approach used requires the following: a method for obtaining the individual pH-titration curves of the base residues; the dissociation constants of cytosine, guanine and uracil residues in the helical and unfolded forms of RNA, which are necessary to estimate the fractions of these residues in helical and amorphous regions; the difference in the spectrum of the helical and unfolded forms of poly(A+U) and polyGC at different pH values; the dependence of the stability of the ordered form of polyGC on pH and ionic strength.

EXPERIMENTAL

Materials

TMV RNA was isolated by extraction of TMV (a gift from Dr R. Markham) with aqueous phenol. Reticulocyte ribosomal RNA was isolated by a modification (Cox, 1966b) of the method of Cox & Arnstein (1963). Crystallizable fragments of yeast RNA (Spencer & Poole, 1965) were a gift from Dr G. L. Brown. *Escherichia coli* RNA was isolated by the method of Littauer & Eisenberg (1959). The properties of polyA and polyU were reported previously (Cox, 1963a). PolyAGUC (AMP:CMP:GMP:UMP proportions approx. 1:1:1:1) was a gift from Dr M. Grunberg-Manago. PolyGC (GMP:CMP ratio 48:52) was a gift from Professor J. Fresco. CMP was obtained from California Biochemical Corp., Los Angeles, Calif., U.S.A. The DNA samples were isolated by Professor J. Marmur by a method described previously (Marmur, 1961). [^{14}N , ^{15}N]DNA was isolated from *E. coli* that was grown for several generations in a medium in which ^{15}N was the sole source of nitrogen and then transferred to a growth medium containing the ordinary ^{14}N isotope, and harvested after one generation as described by Meselson & Stahl (1958). Samples of oligoC and oligoG, which were given by Dr A. Michelson, were fractionated on a Sephadex G-50 column and the faster-moving fractions were used in the titration studies.

Methods

Spectrophotometric measurements. The principal instrument used was a Unicam SP.700 recording spectrophotometer and the temperature was regulated by an electrically heated copper block that also served as a cell holder (Cox, 1963a). The stoppered cuvettes that were used were weighed before and after heating to determine the loss (1% or less) due to evaporation. A correction was also applied for the decrease in extinction due to changes in concentration as a result of the thermal expansion of water. A Beckman model DK-2 recording spectrophotometer was also employed.

Various titration procedures were tried (cf. Cox & Littauer, 1963) but the method finally adopted was to measure the pH of the solution in the cuvette by means of a micro-electrode measuring the range pH 0-14 (Jena Glass combined electrode, 02779 type U; Jenaer Glasswork, Schott und Gen., Mainz, Germany) that was used in conjunction with an E.I.L. model 39A pH-meter (Electronic Instruments Ltd., Richmond, Surrey).

Caesium chloride gradient centrifuging. The procedure followed was essentially that of Meselson, Stahl & Vinograd

(1957). The optical density of the photographic film was measured with a double-beam recording micro-densitometer (model E12 Mk. IIIB; Joyce, Loeb and Co. Ltd., Newcastle upon Tyne).

DEFINITIONS

α_A , α_C , α_G and α_U refer to the degree of ionization, α , of adenine, cytosine, guanine and uracil residues respectively.

$\epsilon_{(P)260}$ refers to the extinction per g. atom of P at 260 m μ . E_{260} , E_{280} etc. refer to the observed extinctions at 260 m μ , 280 m μ etc. $\Delta E_{260}^{\text{denat.}}$ is the total increment in E_{260} due to denaturation. ΔE_{260}^{A-U} etc. is the total increment in E due to denaturation of the base pairs indicated at the wavelength specified. ΔE_{260}^I etc. is the total increment in E_{260} etc. due to ionization. $\Delta E_{260}^{I(A)}$ etc. is the total increment at the wavelength indicated due to ionization of the base residue specified. $\Delta(E_{280}/E_{260})_I$ etc. is the total increment in (E_{280}/E_{260}) etc. due solely to ionization of base residues. $\Delta(E_{280}/E_{260})^{\text{denat.}}$ etc. indicates the total increment in (E_{280}/E_{260}) etc. due solely to denaturation, e.g. by raising the temperature so as to 'melt' the ordered form of a polynucleotide.

f is the fraction of residues in amorphous regions of a polynucleotide. f^0 is the value of f when secondary structure is maximum. f_A , f_C , f_G and f_U respectively are the fractions of adenine, cytosine, guanine and uracil residues in amorphous regions. f_A^I etc. are the fractions of adenine residues etc. in amorphous regions when secondary structure is maximum.

ΔH_i is the heat of ionization of a titratable group.

pH values were based on values of pH 4.01 at 25° for 0.05M-potassium hydrogen phthalate and pH 9.18 at 25° for 0.05M-sodium borate. h is antilog(pH). pH_m is the pH at which 50% denaturation is observed on titration with either acid or alkali at constant temperature. pK_H and pK_{am} refer respectively to the pK (the negative logarithm of the dissociation constant) of a base residue in the helical and amorphous forms of a polynucleotide.

T_m is the temperature at which 50% of the total increase in extinction due to denaturation is observed.

ASSUMPTIONS AND HYPOTHESES

(1) A base residue, whether in a helical or an amorphous segment of a polynucleotide, behaves as a weak monobasic acid and ionizes according to the equation:

$$\text{pK} = \text{pH} + \log[(1-\alpha)/\alpha] \quad (1)$$

This relation has been established for amorphous polyU (Warner & Breslow, 1958).

(2) For a mixture of AMP, CMP, GMP and UMP, α_C may be derived from the equation:

$$\alpha_C = [(E_{280}/E_{260})_{\text{pH}2} - (E_{280}/E_{260})_{\text{pH}7}] / \Delta(E_{280}/E_{260})_I \quad (2)$$

The validity of this equation is discussed in Appendix 1. The greatest error is likely to be found for α_C values of about 0.5. Values in the range 0.5-0.6, depending on the approximations used, may be obtained when the true α_C value is 0.5. Such variation is probably within experimental error.

(3) The change in $\epsilon_{(P)}$ at any wavelength due to ionization

Fig. 1. Difference spectra of the nucleotides in acidic and alkaline solutions and the denaturation spectra of A-U and G-C base pairs in neutral, acidic and alkaline solutions. (a) Difference spectrum for CMP, per g. atom of P ($\epsilon_{(pH 1)} - \epsilon_{(pH 7)}$). (b) Difference spectrum for GMP, per g. atom of P ($\epsilon_{(pH 12)} - \epsilon_{(pH 7)}$). (c) Denaturation spectrum for G-C base pairs, per g. atom of P: —, in neutral solutions [values of Fresco *et al.* (1963) $\times 1.28$ (see the text)]; ----, in acidic solutions, calculated for the transition from the double-helical form in which the base residues are not ionized to an amorphous form in which all cytosine residues are ionized (see eqn. 10), on the basis of the assumption that the effects of ionization and denaturation on the spectrum are additive; ····, in alkaline solutions, calculated for the transition from the double-helical form (G-C) to an amorphous form in which all guanine residues are ionized (see eqn. 11). (d) Difference curve for AMP, per g. atom of P ($\epsilon_{(pH 1)} - \epsilon_{(pH 7)}$). (e) Difference curve for UMP, per g. atom of P ($\epsilon_{(pH 12)} - \epsilon_{(pH 7)}$). (f) Denaturation spectrum for A-U base pairs, per g. atom of P: —, in neutral solutions (values of Fresco *et al.* 1963); ----, in acidic solutions, calculated for the transition from the double-helical form (A-U) to an amorphous form in which all adenine residues are protonated (see eqn. 8); ····, in alkaline solutions, calculated for the transition from the double-helical form (A-U) to an amorphous form in which all uracil residues are ionized (see eqn. 9).

of a base residue is independent of secondary structure, and is approximately the same as that observed for the nucleotide (see Figs. 1a, 1b, 1d and 1e).

(4) Those wavelengths where the extinction of the neutral and ionized forms of a base residue are equal are identical for helical and amorphous polynucleotides. The difference spectra of the corresponding nucleotides yield approximate values for these isosbestic points.

(5) Both oligo- and poly-nucleotides have a strong tendency to form ordered structures that have a lower $\epsilon_{(p)}$ than the amorphous form. The difference in $\epsilon_{(p)}$ for the helical and amorphous forms over the range 220–300 mμ is termed the denaturation spectrum. The fractional change in extinction on denaturation is not necessarily the same for all wavelengths. Thus, for example, E_{280}/E_{260} may not be the same for the helical and amorphous forms, and the precise value of, for example, $\Delta(E_{280}/E_{260})_{\text{denat.}}$ will depend on the nucleotide composition of the helical segment undergoing denaturation because each base pair is characterized by a particular denaturation spectrum.

The denaturation spectra of G-C and A-U base pairs respectively are given in Figs. 1(c) and 1(f) (see Felsenfeld & Sandeen, 1962; Fresco *et al.* 1963).

(6) The contribution of each base pair to the denaturation spectrum is independent of its neighbours [see Felsenfeld & Hirschman (1965) for an analysis of neighbour-interaction on the denaturation spectrum of DNA].

(7) The effects of ionization and denaturation on the spectrum of a polynucleotide are additive. Hence, for example, titration with acid from pH 7 will lead to a change in E_{280}/E_{260} that is given by the equation:

$$\Delta(E_{280}/E_{260}) = \delta\alpha_C \Delta(E_{280}/E_{260})_1 + \delta f \Delta(E_{280}/E_{260})_{\text{denat.}} \quad (3)$$

If, over the pH range studied, secondary structure remains unchanged, eqn. (3) reduces to eqn. (2).

(8) For a mixture of nucleotides, α_U is given by the equation:

$$\delta\alpha_U = \Delta(E_{230}/E_{260})/\Delta(E_{230}/E_{260})_1 \quad (4)$$

because the extinction of GMP (the only other nucleotide

that ionizes on titration with alkali; Fig. 1b) at 230 and 260 m μ is unchanged on ionization.

(9) For a polynucleotide, α_U is obtained from the equation analogous to eqn. (3):

$$\Delta(E_{230}/E_{260}) = \delta\alpha\Delta(E_{230}/E_{260})_1 + \delta f\Delta(E_{230}/E_{260})_{\text{denat.}} \quad (5)$$

and, over the pH range where there is no denaturation ($\delta f = 0$), this equation reduces to eqn. (4).

(10) It may be shown, empirically, for a mixture of nucleotides, that the ionization of the lactam-lactim group of guanine is given by the equation:

$$\delta\alpha_G = \Delta(E_{270}/E_{260})/\Delta(E_{270}/E_{260})_1 \quad (6)$$

For a polynucleotide, α_G is obtained from the equation:

$$\Delta(E_{270}/E_{260}) = \delta\alpha_G\Delta(E_{270}/E_{260})_1 + \delta f\Delta(E_{270}/E_{260})_{\text{denat.}} \quad (7)$$

which, when $\delta f = 0$, reduces to eqn. (6). There is some support (Cox, 1965b) for eqns. (10) and (11) since, for *E. coli* ribosomal RNA, the sum of the spectrophotometric titration curves of guanine and uracil residues is in good agreement with the composite titration curve obtained electrometrically.

(11) The transition between the helical and amorphous forms of a polynucleotide is a co-operative process and so will be reflected in the pH-titration curve as a region of higher buffering capacity than that observed for monomeric acids. Although stacking forces alone may affect the pK of a base residue and so narrow the pH range over which ionization takes place, this may be a small effect because the transition between this type of ordered structure and the amorphous does not take place co-operatively (Brahms, Michelson & van Holde, 1966; see Appendix 4). Other effects, such as the influence of the charge of the polyion, the interaction of neighbouring charged groups (Katchalsky, Mazur & Spitnik, 1956) or the presence of hydrogen-bonded groups that do not undergo co-operative transitions, will tend to broaden the titration curve, i.e. decrease buffering capacity (Cox, 1961).

(12) Thermal denaturation of A--U or G--C base pairs in acidic or alkaline solutions may be accompanied by a change in the degree of ionization of one or other of the base residues because of the large difference between pK_H and pK_{am} and the co-operative nature of the transitions:

(where -- indicates hydrogen bonds, and aq. refers to water). The maximum effect on the spectrum will be observed for the transition from the double-helical form in which the base residues are not ionized to an amorphous form in which the appropriate residues are all ionized. The de-

naturation spectra for these extreme transitions (Figs. 1c and 1f) were calculated by summing the denaturation spectrum (per g. atom of P) with the difference (per 0.5 g. atom of P in these cases) in the spectrum of the neutral and ionized forms of the appropriate mononucleotide (Figs. 1a, 1b, 1d and 1e).

(13) The extent to which these spectra are approached will depend on the difference in the degree of ionization ($\alpha_{am} - \alpha_H$) of the base residue in amorphous and helical forms at the pH studied, as well as on the heat of ionization of the titratable group. Hence (see Cox, 1966a):

$$d\alpha = \left(\frac{\partial\alpha}{\partial T}\right)_f \delta T + \left(\frac{\partial\alpha}{\partial f}\right)_T \delta f \quad (12)$$

where:

$$\left(\frac{\partial\alpha}{\partial T}\right) = -2.3e^{2.3[A + (B/T) - pH]}(1 + e^{2.3[A + (B/T) - pH]})^{-2} (B/T^2) \quad (13)$$

when A is a constant and $B = 0.43\Delta H_i$; and:

$$\left(\frac{\partial\alpha}{\partial f}\right)_T = [K_H/(h + K_H)] - [K_{am}/(h + K_{am})] \quad (14)$$

Thus it appears possible to account quantitatively for the denaturation spectrum observed at various pH values, provided that sufficient background information is available. Such information was therefore sought.

RESULTS

Effects of pH and temperature on the spectrum of E. coli RNA. The spectrum of *E. coli* ribosomal RNA in 0.08 M-sodium chloride-0.008 M-sodium citrate, pH 7.6, was first recorded at 26° (curve I in Fig. 2a) and then at 83° (curve II in Fig. 2a), when secondary structure is largely eliminated (Cox & Littauer, 1962). The pronounced shoulder at 285 m μ at 26° was no longer apparent at 86°. The difference spectrum ($E_{(II)} - E_{(I)}$) (curve III in Fig. 2a), is the denaturation spectrum; no correction for a change in concentration of RNA due to the thermal expansion of water was applied to these data. The form of curve III in Fig. 2(a) is the same as the denaturation spectrum calculated for a mixture of 0.56 part of the curve — in Fig. 1(c) (the denaturation spectrum of G--C base pairs) and 0.44 part of the curve — in Fig. 1(f) (the denaturation spectrum of A--U base pairs) (see also Cox, 1966a). E_{280}/E_{260} increased from 0.44 at 25° to 0.48 at 83°; the difference of 0.04 (Table 1) is the value of $\Delta(E_{280}/E_{260})_{\text{denat.}}$. $\Delta(E_{230}/E_{260})_{\text{denat.}}$ and $\Delta(E_{270}/E_{260})_{\text{denat.}}$ were obtained in a similar way (Table 2).

When the pH was brought from pH 7.6 (curve I in Fig. 2b) to 2.57 at 26° (curve II in Fig. 2b), E_{265} increased by 21%. The difference spectrum ($E_{(II)} - E_{(I)}$) (curve III in Fig. 2b), is shifted towards longer wavelengths compared with curve III in Fig. 2(a): thus the maximum is displaced from 259 to 285 m μ and a minimum is apparent at 239 m μ (cf. the curve ---- in Fig. 1c), as would be expected

Fig. 2. Effect of pH and temperature on the spectrum of ribosomal RNA from *E. coli*. (a) Spectra at pH 7.6 and 26° (curve I; note the inflexion at 280 mμ) and at pH 7.6 and 83° (curve II), and the difference spectrum ($E_{(83)} - E_{(26)}$) (curve III) (solvent: 0.08 M-NaCl-0.008 M-sodium citrate). (b) Spectra at pH 7.6 and 26° (curve I) and at pH 2.57 and 26° (curve II), and the difference spectrum ($E_{(\text{pH } 2.57)} - E_{(\text{pH } 7.6)}$) (curve III) (solvent: 0.08 M-NaCl-0.008 M-sodium citrate). (c) Spectra at pH 6.88 and 25° (curve I), and at pH 11.57 and 25° (curve II), and the difference spectrum ($E_{(\text{pH } 11.57)} - E_{(\text{pH } 6.88)}$) (curve III) (solvent: 0.094 M-sodium phosphate).

if the effect of ionization (principally of cytosine residues) is superimposed on the denaturation spectrum.

The effects of ionization alone were evaluated

by comparing the spectrum of RNA in the amorphous form (curve II in Fig. 2a) with the (presumably) amorphous and ionized form at pH 2.57 (curve II in Fig. 2b). Thus ionization led to no change in E_{270} , a decrease of 0.092 in E_{235} and an increase of 0.136 in E_{290} , whereas the ionization of CMP (Fig. 1a) leads to a substantial increase in extinction at wavelengths greater than 265 mμ and to a decrease in extinction in the range 220–265 mμ (numerically the increase observed at 290 mμ is slightly larger than the decrease found at 235 mμ). The E_{280}/E_{260} value observed at pH 2.57 at 26° is close to the value calculated for an equivalent mixture of nucleotides (cf. Fredericq, Oth & Fontaine, 1961). The increment of 0.1 in E_{300} observed on titration to pH 2.57 (curve III in Fig. 2b) is due solely to ionization since denaturation does not affect E_{300} (see also below).

Titration of RNA with alkali at 25° from pH 6.88 (curve I in Fig. 2c) to pH 11.57 (curve II in Fig. 2c) led to an increase in extinction over the range 220–286 mμ and to a decrease at higher wavelengths (curve III in Fig. 2c). It may be shown that the changes in the spectrum due to ionization (curve II in Fig. 2c—curve II in Fig. 2a, after correction for the difference in concentration of RNA) are essentially the same as those predicted for an equivalent mixture of nucleotides. Thus curve III in Fig. 2(c) is attributable to the sum of the effects of denaturation (curve II in Fig. 2a) and of ionization of guanine (Fig. 1b) and uracil residues (Fig. 1e).

The changes in the spectrum on titration to less extreme pH values are described below.

Spectrophotometric titration of cytosine residues. Preliminary titrations with model compounds confirmed that the major changes in E_{280}/E_{260} ratio on titration are attributable to ionization of cytosine residues despite the complication that even the short oligonucleotides studied appeared to form ordered secondary structures. Titration of oligoG (4–8 nucleotides in size; Michelson, 1959) over the range pH 2.5–7.0 led to no change in E_{280} or E_{260} (Fig. 3a). There was a small increase in E_{300} below about pH 3.2, indicating the pH range over which guanine residues ionize.

Poly(A+U) was titrated with acid from pH 7 and E_{280} was always in the range 0.130 ± 0.006 (Fig. 3b), E_{300} was zero and E_{260} varied from 0.282 to 0.360 owing to changes in secondary structure, so that E_{280}/E_{260} varied between 0.47 and 0.38.

At low pH values the polynucleotides rearranged to poly(A+A) plus polyU and poly(A+U) was not re-formed until pH 5.3 was attained (see also Cox, 1963a). These conformational changes do not affect E_{280} . When poly(A+U), at pH 7, was denatured by heating, E_{280}/E_{260} decreased from

Table 1. Comparison of the calculated and observed limiting E_{280}/E_{260} values for RNA from different sources in neutral and acidic solutions

		E_{280}/E_{260}			$(E_{280}/E_{260})_{\text{denat.}}$	$(E_{280}/E_{260})_1$
		pH 7		pH 2-3		
		25°	approx. 85°			
Reticulocyte RNA	Calc.	0.56	0.56	0.76	—	0.20
	Obs.	0.49	0.53	0.76	0.04	0.23
<i>E. coli</i> RNA	Calc.	0.48	0.48	0.65	—	0.17
	Obs.	0.44	0.48	0.65	0.04	0.17
Yeast RNA	Calc.	0.48	0.48	0.63	—	0.15
	Obs.	0.44	0.48	0.65	0.04	0.17
TMV RNA	Calc.	0.455	0.455	0.58	—	0.125
	Obs.	0.494	0.49	0.616	0.0	0.126

Table 2. Comparison of the observed and calculated E_{230}/E_{260} and E_{270}/E_{260} values for *E. coli* RNA at pH 12

pH	E_{230}/E_{260}		E_{270}/E_{260}	
	Obs.	Calc.	Obs.	Calc.
7 (25°)	0.404	—	0.763	—
7 (85°)	0.398	0.38	0.796	0.83
12 (25°)	0.50	0.50	0.873	0.863
$\Delta_{\text{denat.}}$	0.007	—	0.031	—
Δ_1	0.12	0.12	0.077	0.033

Fig. 3. Titration of oligoG with acid at 21° and titration of poly(A+U) at 22.5°. (a) Titration of oligoG: ○, E_{260} ($E_{260}(\text{pH } 6.99)$ 0.539); ●, E_{280} ($E_{280}(\text{pH } 6.99)$ 0.313); □, E_{300} ($E_{300}(\text{pH } 6.99)$ 0.064) (solvent: 0.08 M-NaCl-0.005 M-sodium acetate). (b) Titration of poly(A+U): ○, titration with acid from pH 7.0; ●, titration with alkali from pH 2.20; curves I and II refer to E_{260} , and curve III refers to E_{300} .

0.47 to 0.38, confirming that the change from 0.47 to 0.38 observed on titration with acid is due to conformational changes. Thus $\Delta(E_{280}/E_{260})_1$ is 0.00 compared with 0.04 calculated for the titration with acid of an equivalent mixture of nucleotides.

In contrast, the titration of oligoC at 19° with acid (Fig. 4) led to an increase in E_{280} from 0.386 to 0.727, whereas E_{260} varied between 0.44 and 0.41, so that E_{280}/E_{260} increased from 0.85 to 1.78 [$\Delta(E_{280}/E_{260})$ 0.93; cf. 1.11 for CMP], and E_{300} increased by 0.15 as a result of ionization. The pH range over which ionization was observed was far broader than that of a monobasic acid and is indicative of organized secondary structure (cf. polyC; Hartman & Rich, 1965).

Dissociation constant of cytosine residues in amorphous RNA. At 75–85° the secondary structure of ribosomal RNA is largely eliminated so that the changes in E_{280}/E_{260} with pH are due mainly to the ionization of cytosine residues. The results obtained for *E. coli* RNA, reticulocyte RNA and crystallizable fragments of yeast ribosomal RNA are given in Figs. 5(a), 5(b) and 5(c) respectively. In each case the titration curves indicate pK 4.0–4.1 (curves III in Figs. 5a, 5b and 5c). Hence the pK at 25° may be calculated if the heat of

ionization (ΔH_i) is known. The value of ΔH_i for cytidylic acid was found to be 5.2 kcal./mole (the dependence of pK on $1/T$ is given in Fig. 6) and is assumed to be the same for cytosine residues in amorphous polynucleotides. On this basis the pK of cytosine residues in the amorphous segments of RNA (in 0.1 M-sodium phosphate at 25°) is 4.7 ± 0.1 (curves II in Figs. 5a, 5b and 5c) compared with 4.2 for cytidylic acid (Peacocke, 1957); the difference is attributed to the normal polyelectrolyte effect of the negatively charged sugar-phosphate 'backbone' opposing the dissociation of a proton. In preliminary titrations of the amorphous polynucleotide polyUC (UMP: CMP ratio 8:1), a value of 4.5 was obtained for the pK of cytosine residues in 0.1 M-sodium phosphate at 25°. Thus the difference between the calculated curves II and the observed curves III in Figs. 5(a), 5(b) and 5(c) is attributable to secondary structure.

The calculated and observed E_{280}/E_{260} values for RNA from the above-mentioned sources, in

Fig. 4. Titration of oligoC with acid at 18.5°. (a) E_{280}/E_{260} ($E_{280}(\text{pH } 6.60)$ 0.443; $E_{280}(\text{pH } 6.60)$ 0.386); (b) E_{280} ; (c) E_{300} ($E_{300}(\text{pH } 6.60)$ 0.068) (solvent: 0.08 M-NaCl-0.005 M-sodium acetate).

neutral and acidic solutions, are compared in Table 1. The increment in E_{280}/E_{260} due to ionization observed on titration of the RNA samples at about 85° was found to be close to the values calculated for the equivalent mixtures of nucleo-

Fig. 5. Spectrophotometric titration of ribosomal RNA at 25° and 75° or 86°. (a) Rabbit-reticulocyte ribosomal RNA. Curve I, titration at 25° [—, data of Cox & Arnstein (1963); ○, this study (solvent: 0.1 M-sodium phosphate)]. Curve III, titration at 86° [●, this study (solvent: 0.1 M-sodium phosphate); ----, theoretical curves for $pK = \text{pH} + \log(1 - \alpha)/\alpha = 4.1$]. Curve II, titration curve for cytosine residues in the amorphous form of RNA derived from curve III on the basis of the assumption that $pK = \Delta H(1/T_2 - 1/T_1)$ when $\Delta H = 5.2$ kcal./mole. (b) *E. coli* RNA. Curve I, titration at 25° [—, data of Cox & Littauer (1963); ○, this study (solvent: 0.1 M-sodium phosphate)]. Curve II, titration at 75° [●, this study (solvent: 0.1 M-sodium phosphate); ----, theoretical curve for pK 4.1]. Curve II, titration curve for cytosine residues derived from curve III as described in (a). (c) Crystallizable fragments of yeast ribosomal RNA. Curve I, titration at 25° [○ (solvent: 0.1 M-sodium phosphate); —, complete titration curve]. Curve III, titration at 86° [● (solvent: 0.1 M-sodium phosphate); ----, theoretical curve for pK 4.0]. Curve II, titration curve for cytosine residues derived from curve III as described in (a).

Fig. 6. Dependence of pK of CMP on temperature. CMP in 0.1M-sodium phosphate was titrated spectrophotometrically at the temperature indicated and the pK was estimated from changes in E_{260} by using eqn. (1). A complete spectrum was obtained at each pH and isosbestic points were noted at 220 and 265m μ .

Fig. 7. Spectrophotometric titration of *D. pneumoniae* DNA in 0.05M-NaCl at 24°.

tides (cf. Fredericq *et al.* 1961). $\Delta(E_{280}/E_{260})_{\text{denat.}}$ was found to be not more than 25% of $\Delta(E_{280}/E_{260})_i$.

Titration of cytosine residues in helical segments. RNA in the intact double-helical form is not readily available. However, DNA would be expected to behave similarly on titration although the precise pK values of the base residues may be different from those of RNA.

Preliminary experiments with DNA from *E. coli* and from *Diplococcus pneumoniae* showed that, in 0.05M-sodium chloride (Fig. 7) and in 0.15M-sodium chloride–0.015M-sodium citrate, titration with acid hardly affected E_{260} until a critical pH (pH_m) was attained, when there was an abrupt increase in E_{260} over a very narrow pH range (cf. Marmur, Schildkraut & Doty, 1962) owing to

Fig. 8. Buoyant density of *D. pneumoniae* DNA after exposure to various pH values. DNA was exposed to (a) pH 3.25 (native DNA), (b) pH 3.10 (acid-denatured DNA; see Fig. 7) and (c) pH 3.22 (intermediate treatment, whereby 45% of the hyperchromicity was destroyed by exposure to acid at 25°) in 0.05M-NaCl at 24°, neutralized and centrifuged for 24 hr. at 44770 rev./min. in a CsCl gradient in the Spinco model E analytical ultracentrifuge. The micro-densitometer tracings represent the concentration of DNA (ordinate) as a function of the distance from the centre of rotation. [^{15}N]DNA from *Pseudomonas aeruginosa* having a density of 1.742 g./ml. was included for reference.

denaturation. The precise value of pH_m depended on ionic strength and the nucleotide composition of DNA (Marmur *et al.* 1962; Bunville & Geiduschek, 1960; Zimmer & Venner, 1962). Neither the buoyant density, 1.702, at pH 7.6 (Fig. 8) nor the thermal stability at this pH (T_m 85°; cf. Marmur & Doty, 1959) were affected by previously titrating *D. pneumoniae* DNA to pH 3.25 just above the transition range) (see Fig. 7), whereas titration to pH 3.20 or less (just beyond the transition range) led to an irreversible change in the buoyant density from 1.702 to 1.716 (Fig. 8) and in T_m from 85° to 35°, the values noted for a sample that had been thermally denatured and rapidly cooled to prevent strand recombination. After controlled titration to pH 3.22 (see Fig. 7) so that 45% of the increase in extinction that accompanies denaturation was achieved the solution was neutralized. Centrifuging in a caesium chloride gradient revealed two components (Fig. 8) having densities 1.702 and 1.716 corresponding to the native state (possibly enriched with DNA of

higher G+C content than average) and the denatured state. Native *E. coli* DNA ($[^{14}\text{N},^{15}\text{N}]$ DNA hybrid) formed one band of density 1.717 in caesium chloride as reported by Marmur *et al.* (1962). After brief (2–5 min.) exposure at pH 2.50 (or pH 12.50) and subsequent neutralization two bands were noticed of buoyant densities 1.725 and 1.740 (identical with those found on thermal denaturation), indicating that strand separation had been achieved.

Although titration with acid led to a small decrease in E_{260} until pH_m was approached, both E_{280} and E_{300} increased reversibly over the range pH 3.3–7 (e.g. E_{280}/E_{260} increased from 0.54 to 0.61 for *D. pneumoniae* DNA and from 0.566 to 0.695 for *E. coli* DNA). Evidently cytosine residues ionize reversibly over this range although the double-helical structure is intact (cf. Dove, Wallace & Davidson, 1959). DNA rich in G+C (isolated from *Streptomyces viridochromogenes*) was studied more closely because the titration of cytosine residues could be followed over a wider pH range. The results are presented in Fig. 9. E_{280}/E_{260} increased reversibly according to eqn. (1) over the range pH 7–3, but increased abruptly and irreversibly on passing through pH 2.75, when denaturation was observed. The curve $\rightarrow$ in Fig. 9(a) was followed on subsequent back-titration with alkali from pH 2.2. E_{300} values were consistent with the changes noted in E_{280}/E_{260} . Decreasing the concentration of sodium chloride from 0.1 M to 0.05 M led to a small increase in pK (from 3.25 to 3.35) and in pH_m from pH 2.75 to pH 2.95.

Estimation of the fraction of cytosine residues in helical and amorphous segments of RNA. The fraction of residues in helical and amorphous segments can be estimated since pK_H and pK_{am} (respectively the dissociation constants of residues in the two forms) are known to be about 3.0 and 4.7 at 25° in 0.1 M-sodium phosphate. Therefore only the cytosine residues in amorphous segments will be ionized to a measurable extent in solutions of pH greater than 4.3; moreover, the fraction of cytosine residues (f_C^0) in amorphous segments at pH 7 remains unchanged over the range pH 4.3–7.0 because at 25° more acidic solutions are required to bring about conformational changes (e.g. Figs. 11 and 12). Hence eqn. (2) reduces to eqn. (1) over the above-mentioned pH range. At pH 4.7, where $\text{pK}_a = \text{pH}$, an approximate value for f_C^0 may be obtained from the equation:

$$f_C^0 = 2\alpha_A = 2\Delta(E_{280}/E_{260})/\Delta(E_{280}/E_{260})_i \quad (15)$$

f_C^0 may be derived more precisely by finding the best value to fit the titration curve above pH 4.3. The fraction, f_C , of cytosine residues in non-helical segments at lower pH values can also be accurately determined (see Appendix 2).

Fig. 9. Spectrophotometric titration of DNA from *S. viridochromogenes* at 25°. (a) Dependence of E_{280} on pH: $\circ \leftarrow \circ$, titration with acid in 0.08 M-NaCl-0.008 M-sodium citrate; $\rightarrow$, titration with alkali from low pH; ----, titration with acid in 0.05 M-KCl. (b) Dependence of E_{300} on pH on titration with acid in 0.008 M-NaCl-0.008 M-sodium citrate ($E_{280}(\text{pH } 7.42)$ 0.456). (c) Relative increase in E_{260} : on titration with acid from pH 7.42: $\circ \leftarrow \circ$, 0.08 M-NaCl-0.008 M-sodium citrate; ----, 0.05 M-NaCl.

The pK of cytosine residues is not necessarily the same for both DNA and RNA since the pK values for cytidylic acid and deoxycytidylic acid are 4.20 and 4.60 respectively (Peacocke, 1957). PolyGC was therefore titrated (Fig. 10), first in 1 mM-sodium phosphate at both 25° and 75° to determine the complete titration range, as well as $(E_{280}/E_{260})_i$ and $(E_{280}/E_{260})_{\text{denat.}}$, and then in 0.1 M-sodium phosphate, when it was found that E_{260} increased by no more than 4%, indicating that the secondary structure was hardly affected by titration to pH 2.4. (This view was confirmed when the effect of temperature on the spectrum at pH 3.0 was examined; see below.) Although E_{280}/E_{260} increased from 0.52 to 0.75 in 0.1 M-sodium phosphate principally over the range pH 2–4, as might be expected for cytosine residues in helical segments,

Fig. 10. Spectrophotometric titration of polyGC. (a) Dependence of E_{280}/E_{260} on pH: $\triangle-\triangle$, 0.1M-sodium phosphate, at 25° (----, theoretical curve for 25% of residues having $pK 4.7$ and 75% having $pK 3.00$); $\circ-\circ$, 1mM-sodium phosphate, at 25°; $\bullet-\bullet$, 1mM-sodium phosphate, at 75° (----, theoretical curve for a monobasic acid). (b) Dependence of E_{280} on pH: $\triangle-\triangle$, 0.1M-sodium phosphate, at 25°; $\bullet-\bullet$, 1mM-sodium phosphate, at 25°.

the form of the titration curve is not described by eqn. (3) but is probably composite, there being a proportion of residues in non-helical segments ($pK 4.7$ in neutral solutions). After taking into account the titration of cytosine residues not involved in secondary structure the pK of the remaining residues appears to be about 3.0 since, except below $pH 3.0$ when denaturation begins (as shown by the increase in E_{280}), the observed E_{280}/E_{260} values fall on the theoretical curve (Fig. 10a) for 25% of residues of $pK 4.70$ and 75% of residues of $pK 3.0$ (see below). Hence the pK of cytosine residues hydrogen-bonded to guanine residues is possibly slightly lower for RNA than for DNA.

Other examples of this approach are given in Figs. 11 and 12. The spectrophotometric titration curves for TMV RNA are given in Fig. 11 and both E_{280}/E_{260} and E_{300} vary with pH in a similar manner. The titration curve is composite: 26% of the increase in E_{280}/E_{260} is due to residues having $pK 4.7$; E_{280}/E_{260} is the same for helical and unfolded forms of TMV RNA [i.e. $\Delta(E_{280}/E_{260})_{\text{denat.}} = 0.0$], so that it is probable that about 26% of the cytosine residues are in amorphous

Fig. 11. Spectrophotometric titration of TMV RNA. (a) Dependence of E_{280}/E_{260} on pH in 0.08M-NaCl-0.02M-sodium acetate: curve II ($\circ$), experimental values; curve I, theoretical curve for 26% of residues of $pK 4.70$; curve III, theoretical curve for 26% of residues of $pK 4.70$ and 74% of residues of $pK 3.0$. (b) Dependence of E_{300} on pH ($E_{260}(pH 6.53) = 0.583$). (c) Dependence of E_{280} on pH.

segments at $pH 7$. Titration below $pH 4.2$ leads to a hyper-sharp titration curve (cf. curve III in Fig. 11a) as a result of the co-operative transition represented in eqn. (10) which leads to the release of cytosine residues from helical segments.

The random co-polynucleotide polyAGUC was also titrated spectrophotometrically (Fig. 12). The changes in E_{300} (Fig. 12b) largely parallel changes in E_{280}/E_{260} (Fig. 12a). The secondary structure is hardly affected over the range $pH 4.6-7.0$ (Fig. 12c), and about 50% of cytosine residues appear to be located in non-helical segments (Fig. 12a). Titration below $pH 4.6$ leads the breakdown of secondary structure as indicated by the increase of E_{280} and by the hyper-sharp nature of the curve E_{280}/E_{260} against pH. The titration curves obtained in 2mM solutions are included in Figs. 12(a) and 12(c) to show the limiting values for E_{280}/E_{260} and of the relative increase in E_{280} .

Fig. 12. Spectrophotometric titration of polyAGUC at 20°: (a) E_{230}/E_{260} ; (b) E_{300} ; (c) relative E_{260} ; solvent: $\circ$, 0.08 M-NaCl-0.02 M-sodium acetate (E_{260} (pH 6.39) 0.737); $\bullet$, 2 mM-NaCl (E_{260} (pH 5.70) 0.543). In (a) ---- indicates the theoretical curve for 50% of residues titrating with $pK_{4.7}$.

Fig. 13. Titration of oligoG with alkali at 21° (solvent: 0.1 M-NaCl-0.025 M-sodium phosphate): (a) E_{230}/E_{260} ; (b) E_{270}/E_{260} ; (c) relative E_{260} . (d) Dependence of E_{253} on temperature (solvent: 0.1 M-sodium phosphate, pH 7.06).

Spectrophotometric titration of the lactam-lactim group of guanine and uracil residues in model compounds. OligoG was titrated with alkali. The E_{230}/E_{260} ratio, used elsewhere as a measure of the ionization of uracil residues, was found to be independent of pH. However, E_{270}/E_{260} increased from 0.79 at pH 7 (compared with 0.82 for GMP) to 0.97 at pH 11.76 (compared with 0.96 for GMP) (Fig. 13). The degree of ionization of guanine residues (α_G) may be evaluated from eqn. (7). The increase in E_{260} of 10%, which would not be expected for amorphous oligoG, is attributed to a change in secondary structure on titration with alkali. Further evidence for the presence of secondary structure at pH 7 was obtained in a preliminary experiment when E_{253} (solvent: 0.1 M-sodium chloride, pH 7.06) increased by about 10% when the temperature was raised from 35° to 85°. The dependence of E_{270}/E_{260} on pH observed for oligoG is therefore unlikely to be characteristic of guanine residues in the amorphous form of a polynucleotide (see Appendix 4).

The complex poly(A+U) was also titrated with alkali (see Fig. 14). E_{270}/E_{260} was hardly affected except over the titration range pH 10.7–10.9, when

a decrease from 0.76 to 0.73 (Fig. 14b), due to denaturation, was observed. However, E_{230}/E_{260} increased from 0.34 at pH 7 to 0.58 at about pH 12 (Fig. 14c). The titration curve calculated on the basis of the assumption that polyA and polyU were amorphous throughout the range pH 7–12 is also given in Fig. 14(c). The difference in the observed and calculated values of E_{230}/E_{260} at pH 7 is $\Delta(E_{230}/E_{260})_{\text{denat.}}$.

Dissociation constant of guanine (NH-CO group) and uracil residues in amorphous RNA. The pK values of GMP and UMP are identical (Peacocke, 1957). The negatively charged phosphodiester 'backbone' of RNA would be expected to affect the ionization of both guanine and uracil residues to the same extent and so identical pK values would be predicted, namely 9.70 at 25° in 0.2 M-sodium chloride as reported for uracil residues in polyU (Warner & Breslow, 1958). Support for this view was obtained by titrating *E. coli* RNA in 8 M-urea, which reduces secondary structure (see Cox & Littauer, 1962). The spectrophotometric titration curves for guanine and uracil residues (Figs. 5a and 5b respectively) were obtained by measuring E_{270}/E_{260} and E_{230}/E_{260} respectively.

Fig. 14. Titration of poly(A+U) at 22° (solvent: 0.05M-sodium phosphate): (a) $\Delta E_{270}/\Delta E_{260}$; (b) E_{270}/E_{260} ; (c) E_{230}/E_{260} ; (d) ΔE_{260} (●), ΔE_{270} (○) and ΔE_{230} (△). In (c) ---- indicates the hypothetical curve for polyA plus polyU without complex-formation.

E_{260} , which is more sensitive to changes in secondary structure than to ionization (cf. Figs. 1b and 1c and Figs. 1e and 1f), increased by about 6% over the range pH 9.0–10.8 but not in more alkaline solutions. Above pH 10.8 the titration curves (Figs. 16a and 16b), are consistent with $pK_{10.1}$. Compared with the curves calculated by using eqn. (1) for $pK_{10.1}$ (Figs. 16a and 16b), the observed curves are steeper over the pH range where E_{260} was found to increase (Fig. 16c). This anomaly is attributed to the release of guanine and uracil residues, in a co-operative manner, from residual secondary structure as described by eqns. (9) and (11) (cf. Cox & Littauer, 1962). Comparison with the curves obtained in the absence of urea (Cox, 1961) reveals that the addition of urea led to the ionization of guanine and uracil residues at lower pH values and to a closer approach to the titration behaviour of a monobasic acid. Urea is known to

Fig. 15. Denaturation spectrum of poly(A+U) at pH 12.05: curve I, spectrum of poly(A+U) at pH 7 (solvent: 0.1M-sodium phosphate); curve II, spectrum of poly(A+U) at pH 12 (solvent: 0.1M-sodium phosphate); curve III, spectrum of polyA plus polyU at pH 7; curve IV, difference spectrum of II-I; curve V, difference spectrum of III-I.

increase the pK of titratable groups (Levy, 1958), and an estimate of this effect was obtained by titrating GMP spectrophotometrically in 8M-urea, when a value of 10.0 ± 0.1 (cf. Stockx & Vandendriessche, 1963) was found at 25° compared with 9.40 in the absence of urea. It is likely therefore that in the amorphous form of RNA both guanine and uracil residues have the same pK (e.g. 9.70 in 0.2M-sodium chloride at 25°).

Titration of uracil residues in double-helical structures. Curve III in Fig. 15 was calculated for a mixture of polyA and polyU at pH 7 assuming no complex-formation, whereas curve I in Fig. 15 was observed. The difference spectrum (curve V in Fig. 15) is the denaturation spectrum at 25°. Curve II in Fig. 15 was observed when the pH was increased from 7 to 12. The difference spectrum (curve IV in Fig. 15), which has the form predicted in Fig. 1(f), is attributable to both denaturation and ionization of uracil residues.

Ionization of UMP leads to an increase in E_{230} , and to a decrease in both E_{260} and E_{270} , whereas E_{245} is unchanged (Fig. 1e). Denaturation of poly(A+U) leads to an increase in extinction in the range 230–280mμ (Figs. 1f and 15), and E_{230} is

Fig. 16. Spectrophotometric titration of *E. coli* ribosomal RNA at 25° (solvent: 0.1M-sodium phosphate in 7.9M-urea): (a) guanine residues; (b) uracil residues; (c) relative E_{260} . In (a) and (b) ---- indicates the calculated titration curve for pK 10.1 (see eqn. 1).

hardly affected. Hence the changes in E_{230} , E_{260} and E_{270} noticed on titration of poly(A+U) with alkali show that ionization is completely suppressed below pH 10 but is concomitant with denaturation over the range pH 10.4–10.8 since E_{260} and E_{270} both increase abruptly over this range together with E_{230} . At higher pH values E_{230} increases, and E_{260} and E_{270} decrease in the predicted manner for uracil residues ionizing according to eqn. (1).

Estimation of the fraction of guanine and uracil residues in helical and amorphous segments. The proportion of residues in amorphous segments can be calculated because, in the double-helical form of a polynucleotide, ionization of guanine and uracil residues is completely suppressed [cf. titration of DNA with alkali (Cox, 1963a) and titration of poly(G+C) (Pochon & Michelson, 1965)]. Thus the observed degree of ionization at any particular pH is due entirely to residues in amorphous segments. Hence:

$$\alpha_U = f_U h / (h + K_{am}) \quad (16)$$

and:

$$\alpha_G = f_G h / (h + K_{am}) \quad (17)$$

The same limitations apply as for cytosine residues (see Appendix 1). The contribution of changes in the secondary structure of RNA to $\Delta(E_{270}/E_{260})$ and $\Delta(E_{230}/E_{260})$ on titration with

Fig. 17. Difference spectrum ($E_T - E_{(25)}$) obtained by heating polyGC at different pH values (solvent: 1mM-sodium phosphate): (a) pH 3.61 (E_{260} 0.624 and E_{280} 0.513 at 25°); (b) pH 4.32 (E_{260} 0.607 and E_{280} 0.462 at 25°); (c) pH 7.08 (E_{260} 0.597, E_{270} 0.482 and E_{280} 0.311 at 25°); (d) pH 10.30 (E_{260} 0.603 and E_{270} 0.492 at 25°); (e) pH 10.80 (E_{260} 0.614 and E_{270} 0.515 at 25°). T was 45°, 55°, 65° and 75° successively in (a), (b), (c) and (e), and T was 55°, 65° and 75° successively in (d). For clarity the difference spectra obtained at other temperatures were omitted.

alkali is about the same order as for $\Delta(E_{280}/E_{260})$ on titration with acid (see Table 2; cf. Table 1 and Figs. 1c, 1f and 2a). The most unfavourable case is the titration of poly(A+U) with alkali (see Fig. 14) because E_{230}/E_{260} changes substantially on denaturation. The plot of E_{230} against pH is a direct measure of the degree of ionization of uracil residues since E_{230} is unaffected when poly(A+U) is denatured, as shown in Fig. 15 from the difference spectra (curves IV and V). The plot of E_{230}/E_{260} against pH is qualitatively similar and it is evident that, once $\Delta(E_{230}/E_{260})_i$ is known, α_U may be evaluated except in the range pH 10.0–10.8 by means of eqn. (4). Provided that there is a simple measure of the extent of denaturation, α_U may also be evaluated within this range by using eqn. (5). In this case E_{245} depends only on denaturation, so that:

$$\delta f_U = \Delta E_{245} / \Delta E_{245}^{\max} \quad (18)$$

Denaturation of polyGC at constant pH. It was shown above that the denaturation of poly(A+U) with alkali led to changes in the spectrum consistent with the assumption that the effects of denaturation and ionization are additive. Studies of the denaturation of polyGC at various pH values also support this approach.

Fig. 18. Thermal denaturation of polyGC at various pH values (solvent: 1 mM-sodium phosphate): upper curves: $\circ$, $\Delta E_{280}/\Delta E_{260}$; $\bullet$, $\Delta E_{270}/\Delta E_{260}$; lower curves: $\circ$, ΔE_{280} ; $\bullet$, ΔE_{260} ; $\triangle$, ΔE_{270} . (a) pH 3.61 (E_{260} 0.624 and E_{280} 0.513 at 25°); (b) pH 4.32 (E_{260} 0.607 and E_{280} 0.462 at 25°); (c) pH 7.08 (E_{260} 0.597, E_{270} 0.482 and E_{280} 0.492 at 25°); (d) pH 10.3 (E_{260} 0.603 and E_{270} 0.492 at 25°); (e) pH 10.8 (E_{260} 0.614 and E_{270} 0.515 at 25°).

The spectrum of polyGC in 1 mM-sodium phosphate was recorded at temperatures in the range 25–75°. The extinction was corrected for changes in concentration due to the thermal expansion of water and the differences in the values observed at the elevated temperature and at 25° were calculated (Fig. 17).

The differences in the spectra observed for polyGC at pH 7.08 are given in Fig. 17(c) (cf. Fig. 1c). The difference spectrum (see also Fig. 1c) had maxima at about 245 and 280 m μ , and a minimum at about 261 m μ ; $\Delta E_{245}/\Delta E_{260}$ and $\Delta E_{280}/\Delta E_{260}$ were about 1.6; ΔE_{300} was very small. E_{260} increased by about 14%, and E_{280} by about 42% principally over the range 50–75° (Fig. 18c); the plateau values were not observed, indicating that complete denaturation was not achieved.

When the pH was brought to 4.32, the differences in the spectra observed on heating approached in form the calculated spectrum for the co-operative denaturation of G-C base pairs in acidic solutions (see eqn. 10). The maximum at 280 m μ became more pronounced ($\Delta E_{280}/\Delta E_{260}$ 2.1; cf. 1.6 at pH 7.08) whereas the maximum at about 245 m μ was diminished ($\Delta E_{245}/\Delta E_{260}$ 0.8; cf. about 1.6 at pH 7.08); between 225 and 245 m μ the extinction hardly increased at all with temperature, and ΔE_{300} , which was negligible at pH 7.08, was approximately the same as ΔE_{245} . Both ΔE_{260} and ΔE_{280} increased over a slightly lower temperature range than at pH 7.08, indicating little change in the thermal stability of secondary structure.

Thermal denaturation of polyGC at pH 3.61 led to the difference spectra given in Fig. 17(a), which are similar in form to those observed on heating at pH 4.32. $\Delta E_{280}/\Delta E_{260}$ was found to be about 2.4 (Fig. 18a) and a substantial increase in E_{300} was again noted. ΔE_{260} and ΔE_{280} increased over a lower temperature range than at pH 4.32, indicating a decrease in the thermal stability of secondary structure (Fig. 18a).

At pH 2.39 the spectrum was unchanged over the range 25–75°, confirming the absence of secondary structure at this pH, as may be inferred from Figs. 10(a) and 10(b).

It is deduced from Fig. 10(a) that raising the temperature of polyGC at pH 4.32 leads to the transition from a double-helical form in which few cytosine residues are ionized to an amorphous form in which about two-thirds of the cytosine residues are ionized. In contrast, heating a solution at pH 3.61 leads to the transition from an ordered form in which about half the cytosine residues are ionized to an amorphous form in which most of the cytosine residues are ionized. The increase in E_{260} noted on titration from pH 7 to pH 3.61 is another indication of the decreased thermal stability at this pH. The abrupt increase in E_{260} (Fig. 10b) below pH 4 indicates the pH range over which denaturation is observed at 25°. At pH 2.4 denaturation is complete.

It was concluded above (see Figs. 10a and 10b) that titration of polyGC in 0.1 M-sodium phosphate to pH 3.0 led to the ionization of about 50% of the

Fig. 19. Thermal denaturation of polyGC at pH 3.04 (solvent: 0.1M-sodium phosphate): (a) $\Delta E_{280}/\Delta E_{260}$; (b) E_{260} (●) and E_{280} (○) (E_{260} 0.600 and E_{280} 0.483 at 25°).

cytosine residues, and this suppression of ionization was attributed to the persistence of secondary structure. The difference spectra observed (cf. Fig. 17b) on raising the temperature at this pH support this view. The maximum at 280m μ was enhanced and $\Delta E_{280}/\Delta E_{260}$ was found to be 2.2 (Fig. 19a). Both E_{260} and E_{280} increased principally above 50° and were still rising at 75° (Fig. 19b).

When solutions of polyGC in 1mM-sodium phosphate were made alkaline, raising the temperature led to changes in the spectrum consistent with those predicted (Fig. 1c) for the co-operative transition given by eqn. (11). When the pH was increased to 10.3 and the solution heated, the difference spectra given in Fig. 17(d) were observed; the maximum was displaced to about 275m μ ; $\Delta E_{270}/\Delta E_{260}$ increased from 1.4 at pH 7.08 (Fig. 18c) to 1.73 (Fig. 18d) and $\Delta E_{245}/\Delta E_{260}$ was slightly diminished, whereas E_{230}/E_{260} and E_{280}/E_{260} were unchanged (for GMP, E_{230} , E_{260} and E_{280} are independent of the ionization of guanine residues) in accord with the trend predicted. The temperature range over which denaturation was observed,

denoted by ΔE_{260} and ΔE_{270} , was about the same as at pH 7.08 (Fig. 18d). Thermal denaturation at pH 10.8 led to difference spectra (Fig. 17e) that approached the calculated denaturation spectrum (Fig. 1c).

DISCUSSION

Titration of model compounds. Oligonucleotides appear to form weak secondary structures (cf. Michelson, 1959). OligoG has both the typical melting profile (Fig. 13d) and pH-titration curve (Fig. 13b) of ordered secondary structure. The stability of secondary structure is appreciably less than that of the self-complexes formed by polyG (Pochon & Michelson, 1965).

OligoC appears to form ordered structures in acidic solutions since the pH-titration curve (Fig. 4a) more closely resembles that of a dibasic acid than that of weak monobasic acid. It is inferred from the titration curve of polyC (Hartman & Rich, 1965) that the uptake of 0.5 proton/nucleotide is sufficient to fully stabilize secondary structure. The uptake of further protons is resisted since the remaining cytosine residues become ionized only when the pH is brought to 3.0. The titration curve (Fig. 4a) has these same characteristics of breadth and shape, although the transitions are not so clearly apparent as for polyC [the dependence of E_{260} on pH (Fig. 4b) is also similar to that noted for polyC]. The energy of interaction between neighbouring cytosine residues may be estimated from the difference in pK values for the first and second 50% of residues titrated (Lifson, 1958) and is about 1 kcal./mole at 20°. It is inferred from the extensive data of Steiner & Beers (1959) that the titration curve of poly(A + A) is also dibasic in character, and that at 26° the energy of interaction between adenine residues is also about 1 kcal./mole.

In contrast, in the double-helical form of DNA, cytosine residues titrate as a weak monobasic acid (see eqn. 1). Hence in this case the ionization of one cytosine residue does not influence the ionization of its neighbours.

The properties of polyGC reported above are consistent with the view (Fresco *et al.* 1963) that secondary structure is formed in which helical and amorphous segments alternate. About 75% of the cytosine residues are involved in helical secondary structure equivalent to 0.39 mole of cytosine/g.atom of P. Hence to place the denaturation spectrum for G-C on a molar basis the values reported by Fresco *et al.* (1963) for this copolymer should be multiplied by 1.28, i.e. 0.50/0.39. The melting temperature (T_m) has been related to the number (N) of G-C base pairs/helical segment by Lipsett (1964), who showed that:

$$T_m = 103 - (174/N) \quad (19)$$

and that short segments with fewer than about 20 base pairs have a lower stability than the infinitely long double-helix (T_m 103–110°). Comparison of the melting range for polyGC (Fig. 18) with Lipsett's (1964) data reveals that the helical segments must be very short, within the range 2–10 base pairs, or grossly imperfect. The melting range of polyAU (see Cox, 1963b) is appreciably lower than that of polyGC, as might be expected on the basis of the known greater stability of G–C base pairs (Marmur & Doty, 1959), and lower than that of poly(A+U), as would be expected if the helical segments of the co-polynucleotide are stabilized by short sequences of less than about 12 base pairs (see Doty, 1961; Lipsett, Heppel & Bradley, 1961).

The secondary structure of the polyAGUC sample studied is characterized by T_m 55° (Cox & Arnstein, 1963) compared with about 37.5° for polyAU (Cox, 1963b), 80° for polyGC and about 95° (Montagnier & Sanders, 1963) for the authentic double-helical form of RNA having the approximate A:U:G:C molar proportions 1:1:1:1. It is inferred from this comparison of T_m values that the sequences of base pairs occurring in polyAGUC are very short, probably in the range 4–8 base pairs. Eqn. (19) may be extended (Cox, 1966a) to any polynucleotide thus:

$$T_m = T_{m(\infty)} - (172/N) \quad (20)$$

where $T_{m(\infty)}$ is the T_m of the authentic double-helical form of a highly polymerized polynucleotide having the same average nucleotide composition.

The titration of polyAGUC with acid (Fig. 12) indicated that about half the cytosine residues were involved in secondary structure and that denaturation took place over the range pH 2.8–5, as might be expected if secondary structure is weak. In this respect polyAGUC (which has a random nucleotide sequence; Ortiz & Ochoa, 1958) is similar to crystallizable fragments of yeast ribosomal RNA. In contrast with polyAGUC, a greater proportion of cytosine residues are involved in more stable secondary structure of *E. coli* RNA and reticulocyte ribosomal RNA, and denaturation was found to take place over a narrower pH range on titration of TMV RNA, which also denatures over a narrow temperature range at pH 7 (Boedtker, 1960). These observations are consistent with the view (Cox, 1966a) that the secondary structure of undegraded ribosomal RNA does not arise from a random nucleotide sequence.

Thermal denaturation at constant pH. The differences in the denaturation spectra of A–U and G–C base pairs are exaggerated when denaturation takes place in acidic or alkaline solutions. In acidic solutions, the $\Delta E_{280}/\Delta E_{260}$ ratio is close to zero for poly(A+U) but increases from about 1.6

to about 2.4 for polyGC (Figs. 18a, 18b and 19). In alkaline solutions the $\Delta E_{270}/\Delta E_{260}$ ratio has the same value as at pH 7 (Fig. 14a) on denaturation of poly(A+U) but increases from 1.4 at pH 7 to 1.73 at pH 10.3 (Fig. 18d) on denaturation of polyGC. Thus it is easier to distinguish the temperature ranges over which double-helical segments differing in nucleotide composition denature by studying denaturation in acidic or alkaline solutions (Cox, 1965a, 1966a).

Criteria for denaturation of A–U and G–C base pairs. There is increasing evidence that the denaturation spectrum observed on heating a neutral solution of polynucleotide is difficult to interpret unambiguously. First, it appears that interaction between adjacent base residues along a single polynucleotide chain may lead to the formation of ordered regions that have the same optical properties as the analogous double-helical form, namely, circular dichromism and optical rotatory dispersion are observed, and $\epsilon_{(P)}$ is lower in the ordered than in the amorphous form at about 25° but increases on raising the temperature (Fasman, Lindblow & Grossman, 1964; van Holde, Brahms & Michelson, 1965; Brahms *et al.* 1966; Holcomb & Tinoco, 1965). Measurements of mass/unit length (Witz & Luzzatti, 1965) also provide evidence for a conformation due to base residues stacked parallel along a single polynucleotide chain.

Secondly, the denaturation spectra of complementary and non-complementary base pairs are similar. The denaturation spectra, at about pH 7, of G–C base pairs (Fresco *et al.* 1963; Pochon & Michelson, 1965), poly(G+G) (Pochon & Michelson, 1965) and polyC (Helmkamp & Ts'o, 1962) are all similar; the denaturation spectra of poly(A+U) and polyA in neutral solutions are also similar (Helmkamp & Ts'o, 1962).

Thirdly, the possibility has not been eliminated that non-complementary base pairs may be accommodated in short helical segments. The available evidence does not favour this view (Fresco & Alberts, 1960; Bautz & Bautz, 1964; see also Lipsett *et al.* 1961; Lipsett, 1964).

It is possible, on the basis of this study, to eliminate some of these ambiguities. First, the transition between an ordered form, stabilized solely by stacking forces, and an amorphous form does not appear to be a co-operative process (van Holde *et al.* 1965; Brahms *et al.* 1966). Thus, if the pK of the base residues in ordered segments of the single strand are different from the pK in the amorphous form, the slope of the pH–titration curve of the residue will be no more than twice that of a monomeric acid and the mid-point may be shifted to some extent (Appendix 4).

Secondly, the denaturation spectrum observed at pH 7 may be supplemented by data obtained at

other pH values. A study of the dependence of T_m on pH is informative. Intuitively, T_m would be expected to depend on pH to an extent governed by the pK values of the residues involved in ordered structure.

Uracil residues have one titratable group (for UMP pK 9.40) so that the stability of poly(U+U) will be unaffected by titration with acid whereas titration with alkali will lead to the elimination of secondary structure. Adenine and cytosine residues each have one titratable group (for AMP pK 3.70, for CMP pK 4.24) so that the secondary structure of polyA and polyC, which is stable at pH 7 (and the hypothetical base pair A--C), will be unaffected by titration with alkali, but will be considerably modified on titration with acid. Guanine residues have two titratable groups (for GMP, pK_1 2.30, pK_2 9.40) so that the stability of poly(G+G) will be decreased by titration to an extreme acidic pH (probably less than 3; cf. Fig. 2) and on titration with alkali (Fig. 13). The stability of the hypothetical base pairs G--U, A--G and C--U would also be affected by titration to the extremes of pH. Likewise, the classical complementary base pairs form hydrogen bonds between the $N_{(1)}=C_{(6)}-NH_2$ group of either adenine (for AMP pK 3.70) or cytosine (for CMP pK 4.24) and the $NH-CO$ group of either uracil (for UMP pK 9.4) or guanine (for CMP pK 9.4) so that titration with either acid or alkali will lead to the elimination of secondary structure (see Fig. 20).

However, the forms of the denaturation spectra will be different at the extremes of pH for each of these base pairs. The calculated denaturation spectra for A--U and G--C base pairs are given in Figs. 1(c) and 1(f). The denaturation spectrum of G--G base pairs will be different from that of G--C base pairs in both acidic and alkaline solutions.

Fig. 20. Phase diagrams for polyGC in 1M-sodium phosphate (curve I) and for polyAU (curve II) in 0.1M-sodium phosphate (Cox, 1963b).

The co-operative transition in acidic solutions is:

and it is apparent from Fig. 3(a) that ionization is observed below pH 3; and in alkaline solutions:

and in both cases $\Delta E_{280}/\Delta E_{260}$ will be independent of pH. The denaturation spectra, calculated on the basis of the assumption that the effects of ionization and denaturation on the spectrum are additive, for the hypothetical base pairs G--U, G--A and C--U (which might occur if irregular double-helical segments are formed) are distinct, as, for example, for the transitions:

and:

Hence, to establish that complementary base pairs stabilize secondary structure it is required to show (a) that in helical segments the number of guanine and cytosine residues are equal; (b) that the number of adenine and uracil residues are equal; (c) that the number of guanine residues characterized by $pK \geq 9.4$ that undergo a co-operative transition on titration with alkali is equal to the number of cytosine residues characterized by $pK \leq 4.24$ that undergo a co-operative transition on titration with acid; (d) that titration with acid or alkali leads to the appearance of the denaturation spectrum of G--C base pairs over pH ranges where the titration curves of guanine and uracil residues are hyper-sharp, leading to the complete elimination of the secondary structure involving G--C base pairs initially present at pH 7; (e) that the number of adenine residues characterized by $pK < 3.70$ that undergo a co-operative transition on titration with acid is equal to the number of uracil residues characterized by $pK \geq 9.40$ that undergo a co-operative transition on titration with alkali and (f) that titration with either acid or alkali leads to the elimination of A--U base pairs and to the appearance of the

A-U denaturation spectrum over the pH ranges where the curves of adenine and uracil residues are hyper-sharp.

The results presented above show that it is possible to derive the spectrophotometric titration curves of cytosine, guanine and uracil residues. The curves so obtained provide at least approximate values for the distribution of these residues in helical and amorphous segments, and of the pH ranges over which denaturation occurs. The data for cytosine and uracil residues are relatively precise. The accuracy of the titration curve of cytosine residues may be improved by following changes in E_{265} and E_{280} , provided that K_H and K_{am} are known (Appendix 2a) rather than E_{280}/E_{260} (Appendix 2b). The data for guanine residues are subject to the greatest error. However, an alternative approach based on observations of ΔE_{230} and ΔE_{280} may provide more precise data (Appendix 3). A limitation of this approach is that the ionization of adenine residues cannot be followed spectrophotometrically.

It is likely that the complementary approach, denaturation at constant pH, may yield the required information provided that the effects of ionization and denaturation on the spectrum are additive. The results described above show that there are wavelengths where the extinction of a base residue is unaffected by ionization, and others where the extinction is unaffected by denaturation of either A-U or G-C base pairs. The precise values of these wavelengths may have to be revised on the basis of further studies with polynucleotides, and the following equations may require revision.

Values of ΔE^i and $\Delta E^{\text{denat.}}$ are required and may be obtained, for example, from the spectra analogous to those given in Fig. 2, although in some cases solvents of lower electrolyte concentration may have to be used to achieve complete denaturation. Now:

$$\Delta E^{\text{denat.}} = \Delta E^{A-U} + \Delta E^{G-C} \quad (25)$$

but at 230 and 280 m μ ΔE^{A-U} is very small so that:

$$\Delta E_{230}^{\text{denat.}} = \Delta E_{230}^{G-C} \quad (26)$$

and:

$$\Delta E_{280}^{\text{denat.}} = \Delta E_{280}^{G-C} \quad (27)$$

Thus, if the shape of the denaturation spectrum of G-C base pairs is known, ΔE^{G-C} may be evaluated at all other wavelengths, e.g.:

$$\Delta E_{265}^{G-C} = (\Delta \epsilon_{265}^{G-C} / \Delta \epsilon_{280}^{G-C}) E_{280}^{G-C} \quad (28)$$

If:

$$\Delta \epsilon_{265}^{G-C} / \Delta \epsilon_{280}^{G-C} = m \quad (29)$$

then:

$$\Delta E_{265}^{G-C} = m \Delta E_{280}^{\text{denat.}} \quad (30)$$

In acidic solutions:

$$\Delta E_{265} = \delta f_C \Delta E_{265}^{G-C} + \delta \alpha_C \Delta E_{265}^{i(C)} + \delta f_A \Delta E_{265}^{A-U} + \delta \alpha_A \Delta E_{265}^{i(A)} + \delta f_G \Delta E_{265}^{i(G)} \quad (31)$$

where $\Delta E_{265}^{i(C)}$ etc. refer to the contribution to ΔE_{265}^i of the base residue indicated. However, $\Delta E_{265}^{i(C)}$ and $\Delta E_{265}^{i(A)}$ are both small, and at pH values higher than about 3.5 (in 0.1 M-sodium phosphate) $\delta \alpha_G$ is negligible, so that eqn. (31) reduces to:

$$\Delta E_{265} = \delta f_C \Delta E_{265}^{G-C} + \delta f_A \Delta E_{265}^{A-U} \quad (32)$$

Now:

$$\Delta E_{280} = \delta \alpha_C \Delta E_{280}^{i(C)} + \delta f_C \Delta E_{280}^{G-C} + \delta f_A \Delta E_{280}^{A-U} + \delta \alpha_A \Delta E_{280}^{i(A)} + \delta \alpha_G \Delta E_{280}^{i(G)} \quad (33)$$

which reduces to:

$$\Delta E_{280} = \delta \alpha_C \Delta E_{280}^{i(C)} + \delta f_C \Delta E_{280}^{G-C} \quad (34)$$

because ΔE_{280}^{A-U} , $\delta \alpha_G$ and $\Delta E_{280}^{i(G)}$ are negligible and $\Delta E_{280}^{i(A)}$ is relatively small.

The difference spectrum obtained on denaturation in alkaline solutions depends on the ionization of guanine and uracil residues as well as on the denaturation of A-U and G-C base pairs. Each parameter can be evaluated from the following equations, which were derived on the basis that $\Delta E_{245}^{i(U)}$, $\Delta E_{250}^{i(A)}$ and $\Delta E_{280}^{i(A)}$ are negligible. Hence:

$$\Delta E_{230} = \delta \alpha_U \Delta E_{230}^{i(U)} + \delta f_G \Delta E_{230}^{\text{denat.}} \quad (35)$$

$$\Delta E_{280} = \delta \alpha_U \Delta E_{280}^{i(U)} + \delta f_G \Delta E_{280}^{\text{denat.}} \quad (36)$$

$$\Delta E_{260} = \delta \alpha_U \Delta E_{260}^{i(U)} + \delta f_G \Delta E_{260}^{G-C} + \delta f_U \Delta E_{260}^{A-U} \quad (37)$$

$$\Delta E_{260}^{\text{denat.}} = \Delta E_{260}^{G-C} + \Delta E_{260}^{A-U} \quad (38)$$

$$\Delta E_{260}^{G-C} = n \Delta E_{280}^{\text{denat.}} \quad (39)$$

$$\Delta E_{270} = \delta \alpha_U \Delta E_{270}^{i(U)} + \delta \alpha_G \Delta E_{270}^{i(G)} + \delta f_U \Delta E_{270}^{A-U} + \alpha f_G \Delta E_{270}^{G-C} \quad (40)$$

$$\Delta E_{270}^{\text{denat.}} = \Delta E_{270}^{A-U} + \Delta E_{270}^{G-C} \quad (41)$$

$$\Delta E_{270}^{G-C} = p \Delta E_{280}^{\text{denat.}} \quad (42)$$

$$\Delta E_{245} = \delta \alpha_G \Delta E_{245}^{i(G)} + \delta f_U \Delta E_{245}^{A-U} + \delta f_G \Delta E_{245}^{G-C} \quad (43)$$

$$\Delta E_{245}^{\text{denat.}} = \Delta E_{245}^{A-U} + \Delta E_{245}^{G-C} \quad (44)$$

$$\Delta E_{245}^{G-C} = q \Delta E_{280}^{G-C} \quad (45)$$

By using the same nomenclature as above, n , p and q are analogous to m and are calculated from the denaturation spectrum for G-C base pairs. Thus:

$$n = \Delta \epsilon_{260}^{G-C} / \Delta \epsilon_{280}^{G-C} \quad (46)$$

$$p = \Delta \epsilon_{270}^{G-C} / \Delta \epsilon_{280}^{G-C} \quad (47)$$

$$q = \Delta \epsilon_{245}^{G-C} / \Delta \epsilon_{280}^{G-C} \quad (48)$$

By successive substitutions and rearrangements:

$$\delta f_G = A \Delta_{230} - B \Delta E_{280} \quad (49)$$

$$\delta\alpha_G = C\Delta E_{280} - D\Delta E_{230} \quad (50)$$

$$\delta f_A = \Delta E_{265} - A'\Delta E_{230} + B'\Delta E_{280} \quad (51)$$

$\delta\alpha_A$ cannot be evaluated

$$\delta f_G = F\Delta E_{280} - G\Delta E_{230} \quad (52)$$

$$\delta\alpha_G = [\Delta E_{245} - (\Delta E_{245}^{A-U}/\Delta E_{260}^{A-U})\Delta E_{260} + K\Delta E_{230} + \Delta E_{280}]/\Delta E_{240}^{I} \quad (53)$$

$$\delta f_U = S\Delta E_{260} + M\Delta E_{230} + N\Delta E_{280} \quad (54)$$

$$d\alpha_U = H\Delta E_{230} - J\Delta E_{280} \quad (55)$$

where:

$$A = 1/[\Delta E_{230}^{\text{denat.}} - \Delta E_{280}^{\text{denat.}} (\Delta E_{230}^I/\Delta E_{280}^I)] \quad (56)$$

$$A' = m\Delta E_{280}^{\text{denat.}} A \quad (57)$$

$$B = 1/[\Delta E_{230}^{\text{denat.}} (\Delta E_{280}^I/\Delta E_{230}^I) - \Delta E_{280}^{\text{denat.}}] \quad (58)$$

$$B' = m\Delta E_{280}^{\text{denat.}} B \quad (59)$$

$$C = (1+B)/\Delta E_{280}^I \quad (60)$$

$$D = A/\Delta_{280}^I \quad (61)$$

$$F = 1/[\Delta E_{280}^{\text{denat.}} - \Delta E_{230}^{\text{denat.}} (\Delta E_{280}^I/\Delta E_{230}^I)] \quad (62)$$

$$G = 1/[\Delta E_{280}^{\text{denat.}} (\Delta E_{230}^I/\Delta E_{280}^I) - \Delta E_{230}^{\text{denat.}}] \quad (63)$$

$$H = 1/[\Delta E_{230}^I - \Delta E_{280}^I (\Delta E_{230}^{\text{denat.}}/\Delta E_{280}^{\text{denat.}})] \quad (64)$$

$$J = 1/[\Delta E_{230}^I (\Delta E_{280}^{\text{denat.}}/\Delta E_{230}^{\text{denat.}}) - \Delta E_{280}^I] \quad (65)$$

$$K = \Delta E_{260}^I (\Delta E_{245}^{A-U}/\Delta E_{260}^{A-U}) H - q\Delta E_{280}^{\text{denat.}} G \quad (66)$$

$$L = (\Delta E_{245}^{A-U}/\Delta E_{260}^{A-U}) \Delta E_{260}^I J - q\Delta E_{280}^{\text{denat.}} F \quad (67)$$

$$M = (n\Delta E_{280}^{\text{denat.}} G - \Delta E_{260}^I H)/S \quad (68)$$

$$N = (\Delta E_{260}^I J - nF\Delta E_{280}^{\text{denat.}})/S \quad (69)$$

$$S = 1/(\Delta E_{260}^{\text{denat.}} - H\Delta E_{280}^I) \quad (70)$$

The precision and accuracy with which $\delta\alpha_G$ etc. and δf_G etc. can be obtained by means of these equations is being evaluated.

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APPENDIX 1

For a mixture of mononucleotides the E_{280}/E_{260} ratio is proportional to the degree of ionization (α) of cytosine residues (eqn. 1):

$$\alpha = \frac{\left[\frac{(E_{280} \pm 1\%)}{(E_{260} \pm 1\%)}_{\text{pH } x} - \frac{(E_{280} \pm 1\%)}{(E_{260} \pm 1\%)}_{\text{pH } 7}\right]}{\left[\frac{(E_{280} \pm 1\%)}{(E_{260} \pm 1\%)}_{\text{max.}} - \frac{(E_{280} \pm 1\%)}{(E_{260} \pm 1\%)}_{\text{pH } 7}\right]} \tag{1}$$

(Limiting values of α calculated for an accuracy of $\pm 1\%$ in extinction are presented in Table 2.)

Eqn. (1) is valid if, first, the E_{260} and E_{280} depend only on ionization of cytosine residues, and, secondly, if the decrease in E_{260} found on ionization of cytosine residues is small compared with the increase in E_{280} .

If, at $260\text{m}\mu$, A is the extinction of adenylic acid, guanlylic acid and uridylic acid at pH 1–7, B is the extinction of non-ionized cytosine residues and C is the extinction of ionized cytosine residues, then at pH x , when the degree of ionization of cytosine residues is α :

$$E_{260} = A + (1 - \alpha) B + \alpha C$$

If, at $280\text{m}\mu$, M is the extinction of adenylic acid, guanlylic acid and uridylic acid at pH 1–7, N is the extinction of non-ionized cytosine residues and P is

the extinction of ionized cytosine residues, then at pH x :

$$E_{280} = M + (1 - \alpha) N + \alpha P$$

Thus:

$$\frac{E_{280}}{E_{260}} = y = \frac{[M + (1 - \alpha) N + \alpha P]}{[A + (1 - \alpha) B + \alpha C]} \tag{2}$$

Hence:

$$\alpha = \frac{[y(A - B) - (M + N)]}{[y(B - C) + (P - N)]} \tag{3}$$

Provided that $y(B - C) \ll (P - N)$:

$$\alpha = \frac{[y(A - B) - (M + N)]}{(P - N)} \tag{4}$$

Eqns. (1) and (4) are equivalent.

The value of A decreases from 36.92 to 36.32 (Table 1) and M increases from 14.41 to 15.20 on titration from pH 1 to pH 7 (Table 1), so that little error is introduced by the first assumption. Values of α derived from eqns. (3) and (4) are compared in Table 2, which shows that the error introduced by the second assumption is within experimental error. It is apparent from Table 2 that α may be determined to within ± 0.04 residue ionized/4g.atoms of P compared with an accuracy ± 0.02 equiv. of acid bound/4g.atoms of P found for electrometric titrations.

Table 1. Molar extinctions of nucleotides at 260 and 280 mμ in acidic and neutral solutions

		10 ⁻³ ε						
		AMP		CMP		GMP		UMP
		pH1-2	pH7	pH1-2	pH7	pH0.5-1.2	pH7	pH1-7
260mμ		14.8	15.4	6.21	7.32	11.51	11.52	10.00
280mμ		3.21	2.63	13.00	7.25	7.95	7.75	4.03

Table 2. Comparison of the degree of dissociation (α) of cytosine residues derived from eqns. (1) and (3)

Eqn. (3)	Eqn. (1)		
	Mean	Minimum	Maximum
0.107	0.100	0.069	0.134
0.215	0.200	0.165	0.235
0.510	0.500	0.457	0.575
0.900	0.900	0.851	0.973

APPENDIX 2

(a) Changes in E_{280} brought about by pH and temperatures can be interpreted to yield the fraction of cytosine residues in helical or amorphous segments.

Let f_c be the fraction of cytosine residues in amorphous segments, f_c^0 be the fraction of cytosine residues in amorphous segments at pH 7, $1-f_c$ be the fraction of cytosine residues in helical segments, K_{am} be the dissociation constant of cytosine residues in amorphous segments, K_H be the dissociation constant of cytosine residues in helical segments, α_{am} be the fraction of cytosine residues in amorphous segments and ionized, $f_c - \alpha_{am}$ be the fraction of cytosine residues in amorphous segments and not ionized, α_H be the fraction of cytosine residues in helical segments and ionized, $1-f_c - \alpha_H$ be the fraction of cytosine residues in helical segments and not ionized and h be antilog (pH):

$$\alpha_{am} = f_c h / (h + K_{am}) \quad (5)$$

$$\alpha_H = (1-f_c) h / (h + K_H) \quad (6)$$

$$f'_c = (f_c - f_c^0) / (1 - f_c^0) \quad (7)$$

Let ΔE_{280}^I be the total increase in E_{280} due to ionization, ΔE_{280}^{G-C} be the total increase in E_{280} due to denaturation and ΔE_{280} be the observed increase in E_{280} . On the basis of the assumption that ΔE_{280} is directly proportional to the ionization of cytosine residues (irrespective of their environment) and to the denaturation of G-C base pairs (i.e. the denaturation spectrum of G-C base pairs is independent of neighbouring nucleotide sequences) and that cytosine residues ionize as a weak acid although the precise value of the dissociation constant depends on conformation (cf. eqns. 5 and 6):

$$E_{280} = [f_c h / (h + K_{am})] \Delta E_{280}^I + [(1-f_c) h / (h + K_H)] \Delta E_{280}^I + f'_c \Delta E_{280}^{G-C} \quad (8)$$

f_c may be evaluated if ΔE_{280}^I , ΔE_{280}^{G-C} , K_{am} , K_H and h are known. If the dependence of K on temperature (or ionic strength) is known, eqn. (5) can also be used to obtain the dependence of f_c on temperature (or ionic strength) at constant pH. The denaturation of A-U base pairs may also be followed once the value of f_c is known, since E_{280} is independent of ionization and reflects the denaturation of both A-U and G-C base pairs.

In practice ΔE_{280}^I and ΔE_{280}^{G-C} may be obtained, for example, in the following way. A stock solution of RNA in 0.5 mM-potassium phosphate, pH 7.8, is prepared: one portion is heated to obtain the limiting value of E_{280} (E_1) for the amorphous form, another portion is brought to pH 3 to obtain the limiting value of E_{280} (E_2) of the ionized amorphous form and a third portion is brought to the required ionic strength to obtain the final value of E_{280} (E_3). Hence:

$$\Delta E_{280}^I = E_2 - E_3 \text{ and } \Delta E_{280}^{G-C} = E_1 - E_3$$

Eqn. (8) may also be placed on a molar basis. K_{am} may be deduced from eqn. (8) over the pH range where f_c is constant and equal to f_c^0 and where α_H is negligible, i.e. above about pH 4.5 in 0.1 M-sodium phosphate solutions.

(b) Finally, the equation:

$$\begin{aligned} \Delta(E_{280}/E_{260}) &= [f_c h / (h + K_{am})] \Delta(E_{280}/E_{260})_I \\ &+ [(1-f_c) h / (h + K_H)] \Delta(E_{280}/E_{260})_H \\ &+ f'_c \Delta(E_{280}/E_{260})_{G-C} \end{aligned} \quad (9)$$

is analogous to eqn. (8) but is not exact (see eqns. 3 and 4), although in some cases E_{280}/E_{260} is more conveniently obtained than absolute values of E_{280} .

APPENDIX 3

Let f_U be the fraction of uracil residues in amorphous segments, f_U^0 be the fraction of uracil residues in amorphous segments at pH 7, $1-f_U$ be the fraction of uracil residues in helical segments, f_g be the

fraction of guanine residues in amorphous segments, f_g^0 be the fraction of guanine residues in amorphous segments at pH 7, $1-f_g$ be the fraction of guanine residues in helical segments, f'_g be $(f_g - f_g^0) / (1 - f_g^0)$,

K be the dissociation constant of uracil residues in the amorphous segments, h be antilog (pH), ΔE_{230}^{G-C} be the total increase in E_{230} at pH 7 due to denaturation, ΔE_{280}^{G-C} be the total increase in E_{280} at pH 7 due to denaturation, $\Delta E_{230}^{(U)}$ be the total increase in E_{230} due to ionization of uracil residues and $\Delta E_{280}^{(U)}$ be the total change in E_{280} due to ionization of uracil residues. On the basis of the assumption that changes in E_{230} and E_{280} are directly proportional to the ionization of uracil residues and to the denaturation of G-C base pairs but are independent of the ionization of guanine residues and the denaturation of A-U base pairs, and that guanine and uracil residues in helical segments do not ionize whereas residues in amorphous segments ionize as weak monobasic acids (cf. eqn. 15) then:

$$\Delta E_{230} = \alpha_U E_{230}^{(U)} + f'_G \Delta E_{230}^{G-C} \quad (10)$$

$$\Delta E_{280} = \alpha_U E_{280}^{(U)} + f'_G \Delta E_{280}^{G-C} \quad (11)$$

After eliminating f'_G and rearranging:

$$\alpha_U = A \Delta E_{230} - B \Delta E_{280} \quad (12)$$

where:

$$A = 1/[\Delta E_{230}^{(U)} - \Delta E_{280}^{(U)} - (\Delta E_{230}^I/\Delta E_{280}^{G-C})] \quad (13)$$

and:

$$B = 1/[\Delta E_{230}^I (\Delta E_{280}^{G-C}/\Delta E_{230}^{G-C}) - \Delta E_{280}^I] \quad (14)$$

Since by definition:

$$K = h\alpha_U/(f_U - \alpha_U) \quad (15)$$

f_U may be evaluated.

After eliminating α_U from eqns. (7) and (8) and rearranging it is found that:

$$f'_G = P \Delta E_{280} - Q \Delta E_{230} \quad (16)$$

where:

$$P = 1/[\Delta E_{280}^{G-C} - \Delta E_{230}^{G-C} (\Delta E_{280}^I/\Delta E_{230}^I)] \quad (17)$$

and:

$$Q = 1/[\Delta E_{280}^{G-C} (\Delta E_{230}^I/\Delta E_{280}^I) - \Delta E_{230}^{G-C}] \quad (18)$$

Eqns. (12) and (16) have been derived on the basis of empirical values for ΔE_{230}^I , ΔE_{280}^I , ΔE_{230}^{G-C} and ΔE_{280}^{G-C} for any given solution of RNA (see above). These equations may also be placed on a molar basis.

APPENDIX 4

Calculated pH-titration curve of dinucleotides and oligonucleotides. The pH-titration curve of base residues in segments of a polynucleotide that have a preferred orientation owing to 'stacking' forces may be derived by considering dinucleotides as models, provided that co-operative effects are absent (cf. Brahms *et al.* 1966). The calculations given below show that the slope of the pH-titration curve ($d\alpha/dpH$) could be twice as great as that of a monomeric acid if the pK of a titratable group is substantially different in the 'stacked' and amorphous forms of a polynucleotide.

Any dinucleotide such as NpNp may be present in solution at pH 7, either as an amorphous structure, represented as:

or as an ordered molecule with the base residues having a preferred orientation, represented as:

Formally, the equilibria will be:

The activities of the different species are $a_1 \dots a_6$ as indicated. Now $k_1 = a_2/a_1$ etc.: $a_2 \dots a_6$ may be expressed in terms of $k_1, K_2 \dots K_5$ and a_1 , i.e.:

$$a_2 = k_1 a_1; a_3 = 2a_1 K_2/h; a_4 = 2a_1 k_1 K_3/h;$$

$$a_5 = a_1 K_2 K_4/h^2; a_6 = k_1 K_3 K_5/h^2$$

where $h = [\text{H}^+]$.

Hence the fraction, α_N , of residues ionized is given by the equation:

$$\frac{1 - \alpha_N}{\alpha_N} = \frac{2a_1 + 2a_2 + a_3 + a_4}{a_3 + a_4 + 2a_5 + 2a_6} = \frac{1 + k_1 + K_2/h + k_1 K_3/h}{K_2/h + k_1 K_3/h + K_2 K_4/h^2 + k_1 K_3 K_5/h^2} \quad (20)$$

The value of h when $\alpha_N = 0.5$ is:

$$h^2 = \frac{K_2 K_4 + k_1 K_3 K_5}{1 + k_1} \quad (21)$$

The fraction, f_N , of residues in the amorphous form is:

$$\frac{1 - f_N}{f_N} = \frac{2a_2 + 2a_4 + 2a_6}{2a_1 + 2a_3 + 2a_5} + \frac{k_1 + 2k_1 K_3/h + k_1 K_3 K_5/h^2}{1 + 2K_2/h + K_2 K_4/h^2} \quad (22)$$

It may be assumed that $K_2 = K_4 = K_{am}$ by analogy with UpUp (Michelson, 1959). When $K_1 = 0$ eqn. (20) reduces to:

$$\frac{1 - \alpha_N}{\alpha_N} = \frac{h}{K_{am}} \quad (23)$$

and if $K_3 = K_5 = K_H$, then when $K_1 \rightarrow \infty$ eqn. (20) becomes:

$$\frac{(1 - \alpha_N)}{\alpha_N} = \frac{h}{K_H} \quad (24)$$

when $K_2 = K_4 > K_3 K_1$ and $K_1 > 5$ the pH-titration curve is determined mainly by k_1 and K_2 and will be symmetrical with a slope ($d\alpha/dpH$) that is about twice that of a simple monomeric acid; and $f_N = \alpha_N$ (cf. the titration curve of oligoG; Fig. 13 of main paper).

Titration with acid. The same principles apply on titration of the $N_{(1)}=C_{(6)}-NH_2$ groups with acid, but since the protonated base residue is the undissociated acid the precise equations are more conveniently formulated as follows:

Since:

$$a_2 = K_1 a_1; a_3 = 2ha_1/K_2; a_4 = 2ha_1/K_3; \\ a_5 = ha_1/K_2 K_4; a_6 = kh^2 a_1/K_3 K_5$$

it follows that:

$$\frac{1 - \alpha}{\alpha} = \frac{1 + k_1 + h/K_2 + hk_1/K_3}{h/K_2 + hk_1/K_3 + h^2/K_2 K_4 + k_1 h^2/K_3 K_5} \quad (26)$$

where α is the fraction of residues carrying a proton; and:

$$\frac{1 - f}{f} = \frac{K_1 + 2hk_1/K_3 + k_1 h^2/K_3 K_5}{1 + 2h/K_2 + h^2/K_2 K_4} \quad (27)$$

The value of h when $\alpha = 0.5$ is:

$$h^2 = \frac{1 + k_1}{1/K_2 K_4 + k_1/K_3 K_5} \quad (28)$$

If $K_2 = K_4 = K_{am} = 3 \times 10^{-5}$ and $K_3 = K_5 = K_H = 1 \times 10^{-3}$, e.g. as for cytosine residues, the pH at the mid-point of the titration curve ($\alpha = 0.5$) is 4.0 when $k_1 = 10$, as might be expected for simple 'stacking' forces [cf. 4.16 observed for CpC (2'-5') (A. M. Michelson, personal communication)], whereas co-operative effects will lead to substantially higher apparent values of k_1 (Brahms *et al.* 1966), e.g. values of k_1 in the range 10^2 – 10^4 are obtained when the observed values of K_H , K_{am} and h at the mid-point of titration curves of cytosine residues of ribosomal RNA from *E. coli* (Fig. 5b of main paper), reticulocytes (Fig. 5a of main paper), polyGC (Fig. 10a of main paper) and DNA (Fig. 9a of main paper) at 25° are substituted in eqn. (28).