Thermal Denaturation in Acidic Solutions of Double-Helical Ribonucleic Acid from Virus-Like Particles Found in *Penicillium chrysogenum*

A SPECTROPHOTOMETRIC STUDY

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1. Two species of double-helical RNA isolated from mycelium of *Penicillium chrysogenum* were titrated with acid at 25°C and 95°C (solvent 0.1M-sodium phosphate buffer). At 25°C denaturation occurred at about pH 3. At 95°C in the denatured form cytosine residues titrated as a simple monobasic acid of pK 3.9 compared with $pK \approx 2.5$ for the native form at 25°C. 2. On thermal denaturation in neutral and acidic solutions one species of RNA (38% rG·rC) 'melted' in three distinct stages, equivalent to a mixture of three species, namely one of about 25% rG·rC, another of about 33% rG·rC and a third of about 46% rG·rC: the relative proportions were 0.25:0.35:0.40. 3. On thermal denaturation in acidic solutions the increase in the fraction of ionized cytosine residues concomitant with the 'melting' of rG·rC base pair also affects the spectrum especially at 280 nm and serves to enhance the contribution of rG·rC base pairs at this wavelength. The increment in $\epsilon_{(P)}$ at 280 nm on 'melting' an rG·rC base pair approaches $53501 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ depending on pH, compared with $33501 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at pH 7. In contrast $\epsilon_{(P)}$ at 280 nm is scarcely affected by 'melting' rA·rU base pairs or by the protonization of adenine residues. 4. Changes in the spectrum of *Escherichia coli* rRNA on denaturation in acidic solutions were studied to yield the mole fractions of rA·rU and rG·rC base pairs 'melting' at particular pH values.

The u.v.-absorption spectrum of a polynucleotide depends on conformation. Conformational changes have been monitored by following the extinction within the range 180-300 nm. Analysis of spectral changes found on thermal denaturation in neutral solutions yields the fraction of base residues involved in double-helical secondary structure and the overall nucleotide composition of the double-helical part (Felsenfeld & Sandeen, 1962; Fresco, Klotz & Richards, 1963). Conformation also influences the acid-base properties of the purine and pyrimidine residues. Thus at certain ranges of pH conformational changes affect both the relative orientation of the base residues and the extent to which they bind protons. The aim of the present work is to show that spectral change obtained when RNA is denatured by heating a solution buffered at a constant acidic pH emphasizes the denaturation of rG·rC base pairs (Cox, 1966*a,b*). A quantitative analysis of this effect was made by studying the thermal denaturation over the range pH 3-7 of double-helical RNA isolated from virus-like

particles found in mycelium of *Penicillium chrysogenum* (Cox, Kanagalingam & Sutherland, 1970). The results show that one of the double-helical species studied has a composite 'melting' profile in neutral and acidic solutions equivalent to that of a mixture of double-helical species, namely one of about 25% rG·rC base pairs accounting for about 25% of residues, another of about 33% rG·rC base pairs accounting for about 35% of residues and a third of about 46% rG·rC base pairs accounting for the remaining 40% of residues.

THEORETICAL

The difference spectrum found on thermal denaturation in acidic solutions has a greater contribution from the 'melting' of rG·rC base pairs than at pH 7 owing to a concomitant increase in the fraction of ionized cytosine residues that leads to an increase in extinction at about 280 nm. Neither the melting of rA·rU base pairs nor the protonization of adenine residues appreciably affects the extinction in this part of the spectrum. Quantitative

analysis of the changes in the spectrum found on thermal denaturation of RNA in acidic solutions is given below.

A principal assumption is that each base pair makes a contribution to the denaturation spectrum that is independent of its neighbours (Felsenfeld & Sandeen, 1962; Fresco *et al.* 1963; Cox, 1970b). The increase found on 'melting' a species of double-helical RNA to an amorphous form in neutral solutions is given by eqn. (1)

$$\Delta\epsilon_\lambda = f_{(rA \cdot rU)} \Delta\epsilon_{(rA \cdot rU)\lambda} + f_{(rG \cdot rC)} \Delta\epsilon_{(rG \cdot rC)\lambda} \quad (1)$$

ϵ_λ is the extinction (E units)/g-atom of P of RNA at wavelength λ nm. $f_{(rA \cdot rU)}$ and $f_{(rG \cdot rC)}$ are respectively the mole fractions of rA·rU and rG·rC base pairs. $\Delta\epsilon_{(rA \cdot rU)\lambda}$ and $\Delta\epsilon_{(rG \cdot rC)\lambda}$ respectively are the increments in ϵ_λ when one mol of rA·rU and of rG·rC base pairs 'melt' to an amorphous state. The amorphous state is considered to be attained when increasing the temperature beyond the range where multihelical conformations are stable leads to a change in ϵ_λ of less than $71 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1} / ^\circ\text{C}$ (i.e. ϵ_{260} increases by no more than 1% of the value at 25°C on increasing the temperature by a further 10°C). Poly(U) at 25°C or above provides an example of a polynucleotide in an amorphous state, since the base-residues appear to have no preferred orientations (see, e.g., Simpkins & Richards, 1967).

Thermal denaturation in acidic solutions. Eqn. (1) is not adequate to describe the increment $\Delta\epsilon_\lambda$ found on thermal denaturation in acidic solutions, because changes in the degree of ionization of the base-residues only adenine and cytosine become protonated over the range pH 3–7. Suppose that $f_{(rA)}$ and $f_{(rC)}$ respectively are the mole fractions of adenine and cytosine residues, whether protonated or not; and that $f_{(rAH^+)}$ and $f_{(rCH^+)}$ respectively are the mole fractions of protonated adenine and cytosine residues. The increments in ϵ_λ on protonization of adenine and cytosine residues are $\Delta\epsilon_{rA(i)\lambda}$ and $\Delta\epsilon_{rC(i)\lambda}$ respectively. On the basis of the assumption that the effects of ionization and denaturation on the spectrum are additive, then the increment $\Delta\epsilon_\lambda$ obtained on thermal denaturation at constant pH in acidic solutions is given by eqn. (2):

$$\Delta\epsilon_\lambda = f_{(rA \cdot rU)} \Delta\epsilon_{(rA \cdot rU)\lambda} + \delta f_{(rAH^+)} \Delta\epsilon_{rA(i)\lambda} + f_{(rG \cdot rC)} \Delta\epsilon_{(rG \cdot rC)\lambda} + \delta f_{(rCH^+)} \Delta\epsilon_{rC(i)\lambda} \quad (2)$$

$\delta f_{(rAH^+)}$ and $\delta f_{(rCH^+)}$ respectively are the increments in $f_{(rAH^+)}$ and $f_{(rCH^+)}$ brought about by heating the solutions. We further assume that the increments $\Delta\epsilon_{rA(i)\lambda}$ and $\Delta\epsilon_{rC(i)\lambda}$ are identical with those found on protonization of a mole of the corresponding mononucleotides (see Pabst Circular OR-10); and that the base residues titrate as weak monomeric acids according to eqn. (3a) or its equivalent form (3b):

$$\text{p}K = \text{pH} - \log [(1 - \alpha)/\alpha] \quad (3a)$$

$$\alpha = [\text{H}^+]/(K + [\text{H}^+]) \quad (3b)$$

where K is the equilibrium constant for the reaction, e.g.:

and α is the mole fraction of protonated base residues (α_{AH^+} and α_{CH^+} refer respectively to adenine and cytosine residues). It is further assumed that the heats of

ionization of the base-residues are independent of the conformation of the polynucleotide and are very similar to the values reported for the mononucleotides.

Eqn. (3a) was shown to apply to poly(U) (Warner & Breslow, 1958; Richards, Flessel & Fresco, 1963). It appears that the negatively charged diesterified ribose phosphate backbone of the polynucleotide influences pK but does not alter the shape of the pH-titration curve of the base residues (Peacocke & Lifson, 1956).

K depends on both conformation and temperature. These effects can be resolved by considering first the change in $f_{(rAH^+)}$ or in $f_{(rCH^+)}$ arising from denaturation at constant pH and temperature and then the effect of raising the temperature when the polynucleotide is amorphous. Suppose that the polynucleotide is in the double-helical form at T_1 °C and is amorphous at T_2 °C. Denaturation at constant pH at T_1 leads to an increment in ϵ_λ owing to ionization of cytosine residues given by eqn. (5) where the subscripts T denote temperature and am and dh respectively indicate the amorphous and double-helical conformations:

$$0.5 f_{(rG \cdot rC)} \Delta\epsilon_{rC(i)\lambda} (\alpha_{rCH^+(am)} T_1 - \alpha_{rCH^+(dh)} T_1) = f_{(rG \cdot rC)} Q_\lambda \quad (5)$$

i.e.

$$Q_\lambda \equiv (\partial f_{(rCH^+)}/\partial f_{(rG \cdot rC)})_{\text{pH}, T_1} \Delta\epsilon_{rC(i)\lambda} \quad (6)$$

Raising the temperature from T_1 to T_2 after denaturation leads to an increment in ϵ_λ owing to ionization of cytosine residues given by eqn. (7):

$$f_{(rC)} \Delta\epsilon_{rC(i)\lambda} (\alpha_{rCH^+(am)} T_2 - \alpha_{rCH^+(am)} T_1) = f_{(rC)} P_\lambda \quad (7)$$

i.e.

$$P_\lambda \equiv (\partial f_{(rCH^+)}/\partial T) f_{(rG \cdot rC)} = 0, \text{pH} \Delta\epsilon_{rC(i)\lambda} \quad (8)$$

Thus:

$$\delta f_{(rCH^+)} \Delta\epsilon_{rC(i)\lambda} = f_{(rC)} P_\lambda + f_{(rG \cdot rC)} Q_\lambda \quad (9)$$

Eqn. (10) gives the analogous terms for adenine residues with M_λ and N_λ corresponding to P_λ and Q_λ :

$$\delta f_{(rAH^+)} \Delta\epsilon_{rA(i)\lambda} = f_{(rA)} M_\lambda + f_{(rA \cdot rU)} Q_\lambda \quad (10)$$

By substituting for $\delta f_{(rAH^+)} \Delta\epsilon_{rA(i)\lambda}$ and $\delta f_{(rCH^+)} \Delta\epsilon_{rC(i)\lambda}$ in eqn. (2) and rearranging we obtain:

$$\Delta\epsilon_\lambda - f_{(rC)} P_\lambda - f_{(rA)} M_\lambda = f_{(rA \cdot rU)} (\Delta\epsilon_{(rA \cdot rU)\lambda} + N_\lambda) + f_{(rG \cdot rC)} (\Delta\epsilon_{(rG \cdot rC)\lambda} + Q_\lambda) \quad (11)$$

At 280 nm eqn. (11) simplifies to eqn. (12) because $N_{280} < 7001 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ and $M_{280} < 7001 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, and so are negligible compared with $\Delta\epsilon_{(rG \cdot rC)280} = 33501 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, and P_{280} and Q_{280} , which depend on $\Delta\epsilon_{rC(i)280} = 57001 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$:

$$\Delta\epsilon_{280} - f_{(rC)} P_{280} = f_{(rG \cdot rC)} (\Delta\epsilon_{(rG \cdot rC)280} + Q_{280}) \quad (12)$$

Hence:

$$f_{(rG \cdot rC)} = (\Delta\epsilon_{280} - f_{(rC)} P_{280}) / (\Delta\epsilon_{(rG \cdot rC)280} + Q_{280}) \quad (13)$$

When eqn. (11) is divided by eqn. (12) we have

$$\frac{[\Delta\epsilon_\lambda - f_{(rA)} M_\lambda - f_{(rC)} P_\lambda]}{[\Delta\epsilon_{280} - f_{(rC)} P_{280}]} = A \left(\frac{f_{(rA \cdot rU)}}{f_{(rG \cdot rC)}} \right) + B \quad (14)$$

where:

$$A = (\Delta\epsilon_{(rA \cdot rU)\lambda} + N_\lambda) / (\Delta\epsilon_{(rG \cdot rC)280} + Q_{280}),$$

and

$$B = (\Delta\epsilon_{(rG \cdot rC)\lambda} + Q_{\lambda}) / (\Delta\epsilon_{(rG \cdot rC)280} + Q_{280})$$

Eqn. (14) may be further simplified because at wavelengths close to 220 nm and 265 nm $\Delta\epsilon_{rC(1)\lambda}$ is small and $\Delta\epsilon_{rA(1)\lambda}$ is small at most wavelengths. Hence at about 265 nm eqn. (12) becomes

$$\frac{\Delta\epsilon_{\lambda}}{(\Delta\epsilon_{280} - f_{(rC)} P_{280})} = A' \left(\frac{f_{(rA \cdot rU)}}{f_{(rG \cdot rC)}} \right) + B' \quad (15)$$

where

$$A' = \Delta\epsilon_{(rA \cdot rU)\lambda} / (\Delta\epsilon_{(rG \cdot rC)\lambda} + Q_{280})$$

and

$$B' = \Delta\epsilon_{(rG \cdot rC)\lambda} / (\Delta\epsilon_{(rG \cdot rC)280} + Q_{280})$$

This analysis should apply both to double-helical polynucleotides and to partly double-helical RNA species provided that the polynucleotide approaches the amorphous state at T_2 . A knowledge of K_{am} and K_{dh} for cytosine residues allows $\alpha_{CH^+(am)}$ and $\alpha_{CH^+(dh)}$ to be calculated at any pH value. Previously it was shown that in 0.1M-sodium phosphate solutions $pK_{am(95^\circ)} = 3.9 \pm 0.1$. The heat of ionization of CMP was measured and the same value ($\Delta H = 5.2 \text{ kcal/mol}$) was assumed to apply to cytosine residues. Hence by means of eqn. (16):

$$pK_{T_2} - pK_{T_1} = (\Delta H/R)(1/T_2 - 1/T_1) \quad (16)$$

$pK_{(am)25} \neq 4.7 \pm 0.1$ was obtained. A value of $pK_{(am)25} = 4.5 \pm 0.1$ was obtained by titrating the polynucleotide poly(U,C) (UMP/CMP ratio 8:1) at 25°C, assuming that the conformation is 'amorphous' at this temperature. Values of $\Delta\epsilon_{rA(1)\lambda}$ are small compared with $\Delta\epsilon_{rC(1)\lambda}$, and the contribution of changes in the degree of ionization of adenine residues to the denaturation spectrum was calculated on the basis of the assumption that $pK_{rA(am)} \simeq pK_{rC(am)}$ and $pK_{rA(dh)} \simeq pK_{rC(dh)}$.

RESULTS

As expected, double-helical RNA resembles DNA in its properties in acidic solutions.

Ionization of cytosine residues in native and denatured RNA. The ionization of cytosine residues in native RNA was followed spectrophotometrically by measuring the spectrum of two species of RNA in 0.1M-sodium phosphate buffer at selected pH values. ϵ_{280} depends on both ionization of cytosine residues and on 'melting' of rG·rC base pairs. The dependence of ϵ_{280} on pH is given in Fig. 1 for fraction 1 (51% G+C, $s_{20,w}$ 12.3S). The major increment in ϵ_{280} found between pH 3.1 and pH 2.7 is attributable to denaturation as shown by a concomitant increase of 30% in ϵ_{260} . The spectrum of fraction 1 was scarcely altered when the pH of the solution was changed from 7 to 3.1. The small change in ϵ_{280} that was observed is attributed entirely to ionization of cytosine residues and shows that in the double-helical conformation $pK < 3$ for cytosine residues, the most likely value being about 2.5. This estimate agrees with an earlier upper limit of pK 3.0 (Cox, 1966b) based on the spectro-

photometric titration of the partly double-helical co-polynucleotide poly(G,C).

Similar results were obtained when fraction 2 was titrated [38% (G+C), $s_{20,w} = 7.3S$], except that denaturation was observed at pH 3.1 as shown by an abrupt increase of 22% in ϵ_{260} .

Both fractions of RNA are denatured at 95°C in 0.1M-sodium phosphate buffer. At this elevated temperature both RNA species approach the amorphous form and the tendency of base-residues to 'stack' one-upon-another in single-stranded regions is minimum. Titration with acid (see Fig. 2) yields $pK = 3.9$ for cytosine residues, in accord with previous work (Cox, 1966b).

Denaturation at constant pH. The difference between the spectrum of a sample of RNA at a temperature in the range 25–95°C and that of a sample kept at 25°C was measured. In solutions more acidic than pH 4.5 the thermal stability (expressed as T_m , i.e. the temperature at which half the increment in ϵ_{260} was observed) of the double-helical form was decreased, and within the range pH 2.7–3.1 denaturation took place at 25°C. Fraction 2 was less stable than fraction 1 at all pH values (see Fig. 3). The increment $\epsilon_{280(95^\circ)} - \epsilon_{280(25^\circ)}$ was found to depend on pH (see Fig. 4). The increment at pH 7 is attributed solely to the denaturation of rG·rC base pairs, and the further

Fig. 1. Dependence of ϵ_{280} of fraction 1 (51% rG·rC) on pH (solvent: 0.1M-sodium phosphate buffer). ϵ_{280} depends both on conformation and on the extent to which cytosine residues are ionized. The relatively small increase in ϵ_{280} over the range pH 3–7 indicates that in the double-helical form ionization of cytosine residues is suppressed ($pK \simeq 2.5$). The abrupt increase in ϵ_{280} found on lowering the pH still further indicates 'denaturation' to a single-stranded form.

Fig. 2. Spectrophotometric titration of cytosine residues of fraction 1 (51% rG·rC) at 95°C (solvent: 0.1 M-sodium phosphate buffer). The $\epsilon_{280}/\epsilon_{260}$ ratio provides a measure of the extent to which cytosine residues are ionized (Cox, 1966b). The broken line is the theoretical pH-titration curve of a monobasic acid (see eqn. 1) of pK 3.9.

Fig. 3. Dependence of T_m of fraction 1 and 2 on pH. T_m is the temperature at which 50% of the total increase in ϵ_{260} is attained. $\circ$, Fraction 1 (51% rG·rC); $\square$, fraction 2 (38% rG·rC). The breadth of the 'melting' range is not indicated.

increase noted at lower pH values arises from ionization of cytosine residues, i.e.

$$\alpha = \alpha_{(rCH^+)am\ 95^\circ} - \alpha_{(CH^+)dh\ 25^\circ}$$

The observed increments, $\Delta\epsilon_{280}$, agree with those calculated by using values of $pK_{am(95^\circ)} = 3.9$ and $pK_{dh(25^\circ)} = 2.5$. The corresponding increase in ϵ_{260} ,

Fig. 4. Change in the extent to which cytosine residues are ionized ($\Delta\alpha_{CH^+}$) as a result of heating fraction 1 (51% rG·rC) at constant pH (solvent: 0.1 M-sodium phosphate buffer). $\circ$, Observed values of $\epsilon_{(P), 280}$ found on heating from 25°C to 95°C. —, Values calculated for $\Delta\alpha_{CH^+}$ assuming that for cytosine residues $\alpha = \alpha_{(CH^+)am(95^\circ)} - \alpha_{(CH^+)dh(25^\circ)}$, that pK_{25° is 2.5 and pK_{95° is 3.9 [pK and α are related by eqns. 3(a) and 3(b)], and that, allowing for the denaturation of rG·rC base pairs, $\Delta\epsilon_{280}$ is directly proportional to the mole fraction of cytosine residues ionized (Cox, 1966b).

i.e. $\Delta\epsilon_{260} = \epsilon_{260(95^\circ)} - \epsilon_{260(25^\circ)}$, scarcely altered over the range pH 3.4–7. Consequently the $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ ratio decreased as the pH was lowered. However, the breadth of the 'melting' curve was not affected. Representative 'melting' profiles of fractions 1 and 2 are given in Fig. 5. The observed difference spectra ($\epsilon_{\lambda(95^\circ)} - \epsilon_{\lambda(25^\circ)}$) are compared with difference spectra calculated from reference spectra for rA·rU and rG·rC base pairs according to eqn. (1) and from the difference spectrum $\epsilon_{(cytosine-H^+)\lambda} - \epsilon_{(cytosine)\lambda}$ for CMP. The agreement between the calculated and the observed difference spectra supports the notion that the effects of denaturation and ionization on the spectrum are additive within the limits of experimental error. Eqns. (13), (14) and (15) were used to calculate the mole fraction of rG·rC base pairs and of rA·rU base pairs 'melting' between 25°C and 95°C from values of $\Delta\epsilon_{260}$ and $\Delta\epsilon_{280}$ obtained within the range pH 3.4–7. The results (Fig. 6) were in satisfactory agreement with the chemical composition of these double-helical species, i.e. $f_{G\cdot C}$ was 0.51 ± 0.01 for fraction 1 and 0.38 ± 0.01 for fraction 2, and so provide further evidence for the validity of this approach.

Breadth of 'melting' profile. The denaturation of both fractions 1 and 2 takes place over a range of about 20–30°C, which is much broader than the

Fig. 5. Representative 'melting' profiles of fractions 1 and 2 (solvent: 0.1M-sodium phosphate buffer, pH 3.6). (a) Fraction 1; (b) fraction 2. In both (a) and (b): $\circ$, ϵ_{260} ; $\bullet$, ϵ_{280} .

range found for bacterial DNA. The increase in extinction appears to be stepwise with fraction 2, and comparison of the rates of increase of ϵ_{260} and ϵ_{280} indicates that the ratio of rA·rU base pairs to rG·rC base pairs is not constant throughout the 'melting' range. An alternating copolymer, such as poly(dA·dT) or poly(rA·rU), 'melts' over a narrow temperature range (about 3°C). The shape of the 'melting' profile is not strongly dependent on molecular weight. Many bacterial DNA species 'melt' over a range of about 6°C. The breadth of the profile depends on nucleotide sequence. If there is a significant variation in rG·rC content within the molecule then rA·rU-rich regions would be expected to 'melt' before rG·rC-rich regions, and the 'melting' curve may be

regarded as a cumulative plot of the 'melting' of regions of different average nucleotide composition. Moreover, RNA isolated from a particular source may not be a single molecular species, so that intermolecular heterogeneity is also to be expected. Thus the 'melting' range is largely a measure of the extent to which there is divergence from the average nucleotide composition owing to intramolecular and intermolecular heterogeneity. Factors influencing the breadth of the 'melting' profile were reviewed by Mandel & Marmur (1968).

The nucleotide composition of independently 'melting' regions may be estimated from the difference spectrum or from $(\epsilon_{260}(T_2) - \epsilon_{260}(T_1)) / (\epsilon_{280}(T_2) - \epsilon_{280}(T_1))$ by means of eqn. (1) (both T_1 and T_2 are temperatures within the 'melting' range).

Fig. 6. Difference spectra observed on heating (a) fraction 1 and (b) fraction 2 in 0.1 M-sodium phosphate buffer, pH 3.6. (a) Fraction 1: curve 1, difference spectrum calculated for 51% rG·rC by means of eqn. (1) by using values of $\Delta\epsilon_{(rA\cdot rU)260} = 45001 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, $\Delta\epsilon_{(rA\cdot rU)280} \approx 01 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, $\Delta\epsilon_{(rG\cdot rC)260} = 14501 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ and $\Delta\epsilon_{(rG\cdot rC)280} = 33501 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (see Cox, 1970b); curve 2, calculated contribution due to the change in the extent of ionization of cytosine residues (cf. Fig. 4) on the basis of the assumption that $\Delta\alpha_{\text{CH}^+} = 0.6$ [this value was calculated by means of eqn. (3a) when $\text{p}K_{\text{dh}(25^\circ)} = 2.5$ and $\text{p}K_{\text{am}(95^\circ)} = 3.9$] and that the change in the spectrum due to ionization of cytosine residues is the same as that for CMP; curve 3 —, values of $\Delta\epsilon(p)$ obtained by summing curves 1 and 2; ○, observed values ($\epsilon_{95^\circ} - \epsilon_{25^\circ}$). (b) Fraction 2: Curve 1: —, calculated values for 37% rG·rC, 0.112 mol of cytosine residues ionized (see a); ○, observed values ($\epsilon_{95^\circ} - \epsilon_{25^\circ}$); curve 2: ----, calculated for the transition $35^\circ\text{C} - 60^\circ\text{C}$ on the basis of 30% rG·rC and $f_{\text{rG}\cdot\text{rC}} = 0.18$, $f_{\text{rA}\cdot\text{rU}} = 0.42$, $\Delta f_{\text{rCH}^+} = f_{\text{rC}\cdot\text{rCH}^+} - f_{\text{rC}\cdot\text{rCH}^+(\text{dh})}$ and by using values of $\text{p}K_{\text{am}(60^\circ)} = 4.29$ and $\text{p}K_{\text{dh}(35^\circ)} = 2.38$; △, observed values; curve 3, calculated as in (a) for the transition $60^\circ\text{C} - 70^\circ\text{C}$ on the basis of 48% rG·rC and $f_{\text{rG}\cdot\text{rC}} = 0.19$, $f_{\text{rA}\cdot\text{rU}} = 0.21$. Δf_{rCH^+} was calculated by using eqn. (3a) on the basis of $\text{p}K_{\text{am}(70^\circ)} = 4.20$ and $\text{p}K_{\text{dh}(60^\circ)} = 2.09$; □, observed values.

In acidic solutions the same information is obtained by using eqns. (13) and (15) to obtain $f_{\text{rA}\cdot\text{rU}(T_2)}$, $f_{\text{rG}\cdot\text{rC}(T_1)}$, $f_{\text{rA}\cdot\text{rU}(T_2)}$ and $f_{\text{rG}\cdot\text{rC}(T_2)}$. For double-helical DNA the percentage content of dG·dC may be calculated from T_m because the relation between these parameters is well established. Previously a tentative relation was proposed by Kallenbach (1968) for RNA (in 0.15 M-NaCl-15 mM-sodium citrate, pH 7) namely:

$$1/T_m(^{\circ}\text{K}) = 2.95 \times 10^{-3} - 0.53 \times 10^{-3} f_{(\text{rG}\cdot\text{rC})} \quad (17)$$

These methods of analysis were applied to the 'melting' profiles of fractions 1 and 2.

Analysis of the 'melting' profile of fraction 1 by means of eqn. (1) showed that the rG·rC content ranged from 47% for the least stable to 53% for the most stable double-helical parts, compared with the overall nucleotide composition of 51% rG·rC. Such a distribution about the mean is in line with those of many other double-helical nucleic acids. Neither intermolecular nor intramolecular heterogeneity can be excluded. The sedimentation profile of this fraction shows the presence of at least three boundaries (Cox *et al.* 1970), indicating that intermolecular heterogeneity could contribute to the breadth of the 'melting' range.

The 'melting' profile of fraction 2 is not only broad but may be resolved into three parts (see Fig. 7) when ϵ_{260} is continuously monitored on raising the temperature from 25°C to 100°C . The nucleotide composition of the least stable species was estimated by means of eqn. (1) to be about 25% rG·rC, compared with about 33% rG·rC for the species of intermediate stability and with about 46% rG·rC for the most stable species. Similar values for rG·rC content were obtained when the appropriate values of T_m were substituted in eqn. (17). The difference spectra obtained when fraction 2 in 0.1 M-sodium phosphate buffer, pH 3.6, was heated are in accord with those predicted from the results of the analysis of the 'melting' profiles obtained by heating neutral solutions. The observed and simulated difference spectra for the range $35 - 60^\circ\text{C}$ (30% rG·rC) and for the range $60 - 70^\circ\text{C}$ (48% rG·rC) are compared in Fig. 6. The results for fraction 2 are summarized in Table 1.

The finding that the changes in extinction that are obtained on heating fraction 2 are readily reversed on cooling (Cox *et al.* 1970) indicates that the double-helical form renatures. Strand recombination is a bimolecular reaction and the rate of renaturation is a measure of intermolecular heterogeneity (see, e.g., Marmur & Doty, 1961). The fast apparent renaturation of fraction 2 suggests that the number of different molecular species is limited. We cannot yet distinguish between the possibility of there being about three different molecular species corresponding to 25% rG·rC,

Fig. 7. 'Melting' profiles of (a) fraction 1 (solvent: 0.1M-NaCl-mM-EDTA, pH 7) and (b) fraction 2 (solvent 0.15M-NaCl-15 mM-sodium citrate buffer, pH 7) as a function of temperature. Values of E_{260} were not corrected for changes in extinction due to changes in concentration as a result of the thermal expansion of water. At 25°C initial values of E_{260} were 0.82 for fraction 1 and 0.80 for fraction 2. The nucleotide compositions of the species involved in the transitions (see Table 1) are indicated.

33% rG·rC and 46% rG·rC, all sedimenting at the same rate, and the possibility of there being a single RNA species whose nucleotide sequence deviates markedly from the average of 38% rG·rC, or a combination of these possibilities.

DISCUSSION

The results provide further evidence for the double-helical character of RNA isolated from virus-like particles found in the mycelium of *P. chrysogenum*. It appears that the 'melting' profile of fraction 2 is irregular, owing to non-uniformity in the nucleotide composition, since it appears that

some molecules or parts of molecules have nucleotide composition that diverges substantially from the mean. In this respect fraction 2 resembles DNA isolated from certain species of protozoa (Mandel, 1967).

The present work also provides further support for the spectrophotometric approach to the analysis of the secondary structure of single-stranded RNA (Cox, 1966a,b). The difference spectra obtained when rRNA was heated in acidic solutions was shown to be in qualitative agreement with the denaturation of rG·rC and rA·rU base pairs. This work provides the basis for quantitative analysis (see Fig. 8). Eqns. (13) and (15) were used

Fig. 8. Analysis of results for unfractionated *E. coli* rRNA. (a) Comparison of observed and calculated values of $\Delta \epsilon(p)_{280}$. Curve 1, calculated on the basis of the assumption that all cytosine residues ($f_{rC} = 0.23$; hence $f_{rG \cdot rC} = 0.46$) are base-paired at 25 $^{\circ}\text{C}$ but are in an amorphous conformation at 95 $^{\circ}\text{C}$, i.e. $\Delta \epsilon_{280} = 0.5 f_{(rG \cdot rC)} \Delta \epsilon_{rC(1)280}(\alpha_{rC(am)950} - \alpha_{rC(dh)250}) + f_{rG \cdot rC} \Delta \epsilon_{(rG \cdot rC)280}$ (as for CMP, $\Delta \epsilon_{rC(1)280} = 57001 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). Curve 2 was calculated on the basis of the assumption that cytosine residues ($f_{rC} = 0.23$) are in amorphous conformation at 25 $^{\circ}\text{C}$ and 95 $^{\circ}\text{C}$, i.e. $\Delta \epsilon_{280} = f_{rC} \Delta \epsilon_{rC(1)280}(\alpha_{rC(am)950} - \alpha_{rC(am)250})$. Curve 3, observed values (data of Cox, 1966a). It is inferred that over the range pH 4–7 about 75% of cytosine residues are base-paired at 25 $^{\circ}\text{C}$ (i.e. $f_{rG \cdot rC} = 0.36$) and that denaturation takes place at lower pH values. This figure is the graphical equivalent of eqn. (13) and at any pH $f_{rG \cdot rC} = (\Delta \epsilon_{\text{obs.}} - \Delta \epsilon_{(\text{curve 2})}) / (\Delta \epsilon_{(\text{curve 1})} - \Delta \epsilon_{(\text{curve 2})})$. (b) Values of f_{dh} , $f_{rG \cdot rC}$ and $f_{rA \cdot rU}$ as a function of pH calculated from the difference spectra $\epsilon_{(950)} - \epsilon_{(250)}$ (Cox, 1966a) by means of eqns. (13) and (15). (c) Analysis of the difference spectra observed on heating rRNA in 0.1M-sodium phosphate buffer, pH 4.0 (Cox, 1966a), by means of eqns. (13) and (15).

to analyse published results for unfractionated *Escherichia coli* rRNA. Values of f_{dh} , $f_{rG \cdot rC}$ and $f_{rA \cdot rU}$ at 25 $^{\circ}\text{C}$ were calculated as a function of pH from the change in the spectrum found on heating from 25 $^{\circ}\text{C}$ to about 95 $^{\circ}\text{C}$ (see Fig. 8b), and so were the changes in f_{dh} etc. on heating at pH 4 (see Fig. 8c). The results agree with an earlier analysis (Cox, 1966a).

Denaturation in acidic solutions offers the advantage that the contribution to the difference

spectrum over the range 280–320nm of $rG \cdot rC$ base pairs on ‘melting’ is enhanced compared with denaturation at pH 7, whereas the contribution of $rA \cdot rU$ base pairs remains negligible. The plot of T_m against pH (see Fig. 3) and also the plot of $\Delta \epsilon_{280}$ against pH (Figs. 4 and 8a) appear to depend on nucleotide composition, and so may serve to characterize a particular RNA species (cf. Cox, 1966a,b) or fragments of known nucleotide sequence. In essence the chemical reactivity of cytosine

Table 1. *Summary of the properties of fraction 2 inferred from the 'melting' profile*

Solvent	Temperature range (°C)	T_m (°C)	$\Delta\epsilon_{260}/\Delta\epsilon_{280}$	rG·rC content (%)		Approx. % of residues involved in the transition
				Calc. from T_m (eqn. 11)	Calc. from difference spectrum (eqns. 3 or 7 and 9)	
0.15 M-NaCl-15 mM-sodium citrate, pH 7	75-85	80	4.1	23	25	25±2
	86-88	87	3.3	31	33	35±3
	75-88	—	3.6	—	30	60±5
	89-96	94	2.1	44	46	40±5
	75-96	—	2.85	—	37	100
0.1 M-sodium phosphate buffer, pH 3.6	35-60	50	2.06	—	30	60±5
	60-70	63	1.24	—	48	40±5
	35-70	—	—	—	—	100

residues (in the present case with protons) is studied.

Interpretation of the difference spectrum found on denaturation at pH 7 is limited by the contribution of base-residues that are stacked one-upon-another in single-stranded regions of a polynucleotide. The same limitation applies to the analyses of the spectral changes found on denaturation in acidic solutions. 'Stacking' in single-stranded regions affects the pH-titration properties of a base-residue (for discussion see, e.g. Cox, 1966b). The qualitative effects of changes in pH on 'stacking' in single-stranded regions were deduced by using dinucleotides as models on the basis of the assumption that the base residues have either a 'stacked' or 'unstacked' conformation. By means of the schemes given by Cox (1966b) it can be calculated that decreasing the pH from 7 to 5 tends to decrease stacking between Cp-Cp, Ap-Ap, and Ap-Cp and that 'stacking' is minimum at about pH 3 for these dinucleotides. However, for dinucleotides such as Gp-Gp and Up-Gp, and to a lesser extent Ap-Gp and Cp-Gp pH values within the titration range of guanine residues (i.e. less than pH 3) are required before the extent of 'stacking' is decreased. Thus provided that equivalent states of a polynucleotide are compared the extent of base 'stacking' in single-stranded regions will be less in acidic than in neutral solutions. Thus estimates of $f_{rG\cdot rC}$ calculated at say pH 4.5, a pH where even short double-helical regions are stable, are likely to be less affected by 'stacked' but unpaired residues than estimates obtained by denaturation at pH 7. Owing to the low pK of guanine residues 'stacking' in single-stranded regions may persist after the elimination of double-helical secondary structure, e.g. when fraction 1 was denatured by titration from pH 7 to pH 2.7 the increment in ϵ_{260} was about 30% compared with about 44% on heating at 37°C.

The smaller increment found on denaturation at pH 2.7 at 25°C is attributed to 'stacking' involving mainly guanine residues. The results are insufficient to permit quantitative measurements of 'stacking' in single-stranded regions in acidic solutions.

The lower thermal stability of rRNA compared with fractions 1 and 2 is attributed to differences in the length of the double-helical regions (cf. Kallenbach, 1968). These differences in thermal stability were exploited to provide estimates of the number of base pairs per double-helical region of rRNA (Cox, 1966a, 1970a). More extensive knowledge of the properties of model compounds in acidic solutions are required before a similar analysis can be made of results obtained by thermal denaturation in acidic solutions.

EXPERIMENTAL

Materials. Chemical reagents used were of A.R. quality, unless otherwise stated. Polyethylene glycol (mol. wt. 6000) was the Union Carbide Ltd. product Carbowax 6000 obtained from Evans Medical Ltd. (Speke, Liverpool, U.K.).

K_2HPO_4 , 2-methoxyethanol and 8-hydroxyquinoline were of laboratory reagent grade from BDH Chemicals Ltd. (Poole, Dorset, U.K.). To liquified phenol B.P. grade from Evans Medical Ltd. was added 8-hydroxyquinoline to 0.1%. Nipastat, a bacteriostatic agent, was obtained from Nipa Laboratories Ltd. (Treforest Industrial Estate, Treforest, Glam., U.K.).

Column chromatography was carried out on the 100-200 mesh grade of Bio-Gel A-15m (4% sphere-beaded agarose) obtained from Bio-Rad Laboratories (Richmond, Calif., U.S.A.). [In a previous paper (Cox *et al.*, 1970) the Bio-Gel used was described in error as 200-400 mesh].

Methods. Growth of *P. chrysogenum* was as described by Cox *et al.* (1970). The mycelium was separated from the culture fluid by filtration, and the mycelium frozen immediately and stored at -20°C until extracted.

Frozen mycelium (15 kg) was suspended in 40 litres of 50 mM-phosphate buffer, pH 8 (prepared from Na_2HPO_4

and KH_2PO_4), containing 20 g of Nipastat, and allowed to thaw overnight. To release the mycophage from the mycelium, the suspension was passed twice through a Manton-Gaulin homogenizer (type 15M-8BA, from A.P.V. Ltd., Manor Royal, Crawley, Sussex, U.K.). The temperature was initially 4°C, and was not allowed to rise above 25°C.

After the addition of 1 kg of kieselguhr filter-aid, the homogenate was filtered through a 1.5 cm kieselguhr bed on a large Doulton filter. NaCl (to 0.2 M) and polyethylene glycol (to 4%, w/v) were added to the filtrate (42 litres). After being stirred at ambient temperature for 20 min, the mixture was allowed to stand for 1 h. The pH was then adjusted to 5.0 with acetic acid, and the precipitate, containing the mycophage, allowed to settle overnight at 4°C. The greater part of the supernatant was separated by decantation and the precipitate separated from the remaining liquid by low-speed centrifugation for 1 h.

The precipitate was suspended in one-twentieth the original filtrate volume of 0.1 M-sodium acetate, and 4 M-NaOH was used to adjust the pH to 7.0 with continuous stirring. At this stage the mycophage was disrupted and the released nucleic acids were freed from protein by treatment with phenol. The suspended precipitate was mixed very thoroughly for 30 min with an equal volume of phenol solution, and the phases were separated by centrifugation at 2100g for 1 h at 5°C. The aqueous phase, containing the released nucleic acids, was separated and the phenol treatment was repeated once. The nucleic acids were then precipitated from the aqueous phase by the addition of 2 vol. of ethanol. The precipitation was repeated twice from 0.3 M-sodium acetate with an equal volume of ethanol, to free the nucleic acids from phenol.

Removal of polysaccharides. The precipitate was dissolved in water, and all residual ethanol was removed by concentration under vacuum at a temperature not exceeding 30°C. Polysaccharides were removed by the method of Kirby (1956). To 500 ml of aqueous nucleic acid solution were added 500 ml of 2.5 M- K_2HPO_4 , 500 ml of 2-methoxyethanol and 25 ml of 33% (v/v) H_3PO_4 . After a thorough mixing the phases were separated by centrifuging at 2100g for 20 min at 20–25°C. The lower phase was discarded. The upper phase was mixed with 750 ml of ethanol, causing the nucleic acids to precipitate. After centrifugation, the liquid phases were carefully decanted from the precipitate that separated at the interphase. The precipitate was dissolved in water and dialysed at 5°C against 0.01% sodium acetate.

Separation of fraction 1. The dialysed nucleic acid solution, obtained from several extractions as described above, was concentrated to 300 ml (E_{260} approx. 100); 300 ml of 4 M-NaCl was added, followed by 60 ml of ethanol. After being left for at least 18 h at 4°C the solution was centrifuged at 35000g for 1 h at 5°C. A small precipitate, consisting mainly of residual polysaccharides and denatured proteins, was rejected. The temperature of the supernatant solution was allowed to rise to 25°C, and 45 ml of ethanol was added slowly. The solution was stored at 4°C overnight, when a gel-like precipitate of fraction 1 formed, and was separated by centrifuging at 35000g for 1 h at 5°C. The supernatant was decanted off and kept. The precipitate was dissolved in water,

ethanol was removed under vacuum, and the fraction 1 was precipitated as before from 2 M-NaCl by the addition of ethanol to 15% (v/v) (assuming no volume change). Standing at 4°C and centrifuging were as described above. The supernatant was separated and combined with the earlier supernatant.

The pelleted nucleic acid after dialysis and freeze-drying had $E_{1\text{cm}}^{1\%}$ 167 at 260 nm, measured in 0.15 M-NaCl. A 6–7 mg sample was dissolved in 10 ml of 0.2 M-sodium acetate containing 1 mM-EDTA (disodium salt) and loaded on a Bio-Gel A-15m column (8 cm² × 95 cm) packed in the same salt solution. On the basis of the extinction at 260 nm, the sample contained approx. 94% of fraction 1 and less than 4% of fraction 2.

Separation of fraction 2. The supernatants from the two precipitations of fraction 1 were combined, and the nucleic acid was precipitated by the addition of 0.5 vol. of ethanol and separated by centrifugation. The precipitate was dissolved in water, all ethanol was removed under vacuum and the volume was adjusted to 100 ml. Then 100 ml of 4 M-NaCl and 35 ml of ethanol were added, the solution was allowed to stand for 18 h at 4°C, and any remaining fraction 1 was spun off at 35000g for 1 h at 5°C.

After it had been warmed to 25–30°C another 15 ml of ethanol was added to the separated supernatant to raise the ethanol content to a nominal 20% (v/v). The cooling and centrifuging cycle was repeated and the fraction 2-rich precipitate was dialysed and freeze-dried.

Fraction 2 was further purified by loading 30 mg on a Bio-Gel A-15m column packed and run in 0.2 M-sodium acetate. The peak fractions were combined, concentrated, dialysed and freeze-dried. The $E_{1\text{cm}}^{1\%}$ of the solid was 167 at 260 nm. Electrophoresis through 5% polyacrylamide gels (6 mm internal diam. × 6 cm) run at 3 mA/tube for 1 h and then stained with Methylene Blue showed a single band 39–40 mm from the origin (reservoir buffer 5 mM-tris–38 mM-glycine, pH 8.3, gels pre-run for 2.5 h).

Measurement of 'melting' profile. A Pye Unicam SP.1800 double-beam spectrophotometer was used, with a Unicam SP.876 temperature-programme controller and a Unicam SP.877 electrically heated cell holder. The E_{260} value of the fraction 2 sample in standard saline citrate (0.15 M-NaCl–15 mM-sodium citrate, pH 7) was first measured against a standard saline citrate blank. The zero shift mechanism was then operated and the meter adjusted to read zero. The cell block was heated at 1°C/min and the increase in extinction was recorded directly against the rise in temperature by using an X–Y recorder (Auto Plotter model 21000 from Bryans Ltd., Mitcham, Surrey, U.K.). The temperature of the solution was measured by using one of the spare spaces in the eight-cell holder to accommodate the cell in which the temperature-sensing element was immersed.

Measurement of difference spectra. The principal instrument used was a Unicam SP.700 spectrophotometer. The sample-cell holder was an electrically heated copper block that could be maintained at temperatures between 25°C and 95°C. The difference between the spectrum of the heated sample and that of a sample in the reference cell kept at 25°C was measured over the range 220–330 nm. A correction was applied for changes in extinction arising from the expansion of water.

The pH of a solution was measured by means of a micro-electrode serving the range pH0-14 (Activion Glass Ltd., Kinglassie, Fife, U.K.) that was used with an E.K. model 39 A pH-meter (Electronic Instruments Ltd., Richmond, Surrey, U.K.).

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REFERENCES

- Cox, R. A. (1966a). *Biochem. J.* **98**, 841.
Cox, R. A. (1966b). *Biochem. J.* **100**, 146.
Cox, R. A. (1970a). *Biochem. J.* **117**, 101.
Cox, R. A. (1970b). *Biochem. J.* **120**, 539.
Cox, R. A., Kanagalingam, K. & Sutherland, E. (1970). *Biochem. J.* **120**, 549.
Felsenfeld, G. & Sandeen, G. (1962). *J. molec. Biol.* **5**, 587.
Fresco, J. R., Klotz, L. C. & Richards, E. G. (1963). *Cold Spring Harb. Symp. quant. Biol.* **28**, 83.
Kallenbach, N. R. (1968). *J. molec. Biol.* **37**, 445.
Kirby, K. S. (1956). *Biochem. J.* **46**, 405.
Mandel, M. (1967). In *Chemical Zoology*, vol. 1, p. 554. Ed. by Florkin, M. & Scheer, B. T. New York: Academic Press Inc.
Mandel, M. & Marmur, J. (1968). In *Methods in Enzymology*, vol. 12B, p. 195. Ed. by Grossman, L. & Moldave, K. New York: Academic Press Inc.
Marmur, J. & Doty, P. (1961). *J. molec. Biol.* **3**, 585.
Peacocke, A. R. & Lifson, L. (1956). *Biochim. biophys. Acta*, **22**, 191.
Richards, E. G., Flessel, C. P. & Fresco, J. R. (1963). *Biopolymers*, **1**, 431.
Simpkins, H. & Richards, E. G. (1967). *J. molec. Biol.* **29**, 349.
Warner, R. C. & Breslow, E. (1958). *Proc. 4th int. Cong. Biochem., Vienna*, vol. 9, p. 157.