The Secondary Structure of Ribosomal Ribonucleic Acid in Solution

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1. The u.v.-absorption spectrum of ribosomal RNA from rabbit reticulocytes was studied as a function of temperature at different pH values. The changes in the spectrum over the range 220–320 m μ were interpreted on the basis of the assumption that the effect of denaturation and ionization are additive. The results suggest that in neutral salt solutions the secondary structure of the ribosomal RNA samples studied is due to two species of helical segments stabilized principally, if not solely, by complementary base pairs but differing in nucleotide composition: each species appears to be heterogeneous in other respects in view of the breadth of the melting ranges. 2. The number of base pairs per helical segment was estimated to be small (between 4 and 17) on the basis of the relation between melting temperature and chain length previously established by Lipsett and others for model compounds. Small fragments (about 2 s) obtained by alkaline hydrolysis appeared to form the same helical segments as the intact molecule in accord with the estimated size of these segments. 3. Specific nucleotide sequences appear necessary to account for the hysteresis observed on titrating ribosomal RNA with acid or alkali within the range pH 3.0–7.0 since this phenomenon was less pronounced for *Escherichia coli* transfer RNA and for RNA from turnip yellow-mosaic virus.

It appears that within the ribosome the RNA component forms, through intramolecular interactions, a stable helical secondary structure that is retained after dissociation of the protein moiety (for reviews see Arnstein, 1963; Petermann, 1964). A knowledge of the secondary structure of RNA in solution is relevant therefore to understanding the conformation of ribosomal RNA *in vivo*. Although the principal features of the secondary structure of RNA are known (for review see Spirin, 1963) further studies were initiated in an attempt to assess the contribution of specific nucleotide sequences to secondary structure, to identify the base pairs stabilizing helical segments and to estimate the size of helical segments.

The secondary structure of ribosomal RNA was characterized by studying the changes in the absorption spectrum brought about by increasing the temperature at different values of pH and electrolyte concentration. The results obtained on heating in neutral solutions were interpreted on the basis of the work of Felsenfeld & Sandeen (1962) and of Fresco, Klotz & Richards (1963). The changes in the spectrum of RNA noticed on heating at other pH values was interpreted on the basis of other studies (Cox, 1966; see also the Theoretical section). The results were correlated with an earlier approach (Cox, 1961; Cox and Arnstein, 1963) where

the temperature was maintained constant and the pH was varied in order to yield the proportion of residues involved in secondary structure and the pH range over which cytosine, guanine and uracil residues were released from secondary structure.

The results of both types of experiment are consistent with the presence in ribosomal RNA from *Escherichia coli* and reticulocytes of at least two species of helical segments differing in nucleotide composition. The number of base pairs in the less thermally stable helical segments (approx. 50% of G + C) and in the more stable helical segments (approx. 85% of G + C) appears to be small (probably within the range 4–17). It is inferred from a comparison of the electrometric-titration properties of ribosomal RNA, TYMV* RNA and *E. coli* transfer RNA that specific nucleotide sequences give rise to the hysteresis noted on titration of ribosomal RNA over the range pH 3–7.

A short account of this work has been presented (Cox, 1965).

* Abbreviations: TYMV, turnip yellow-mosaic virus; 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.

THEORETICAL

The denaturation spectrum, i.e. the difference in $\epsilon_{(P)}$ (the molar extinction coefficient referred to 1 g. atom of P), over the range 220–300 $m\mu$, found for the thermally induced transition from double-helical to amorphous forms of G--C and A--U pairs, namely:

$$G--C \rightleftharpoons G--aq. + C--aq. + \Delta\epsilon_{(P)}^{GC} \quad (1)$$

$$A--U \rightleftharpoons A--aq. + U--aq. + \Delta\epsilon_{(P)}^{AU} \quad (2)$$

(where -- indicates hydrogen bonds) are distinct (Felsenfeld & Sandeen, 1962; Fresco *et al.* 1963). The denaturation spectrum of G--C base pairs has maxima at 245 and 278 $m\mu$, whereas the denaturation spectrum for A--U base pairs has a maximum at 262 $m\mu$ and both helical and amorphous forms have identical values of $\epsilon_{(P)}$ at 230 and 280 $m\mu$.

The nucleotide composition of helical segments melting to an amorphous form over a particular temperature range has been estimated on the basis of the assumption that each base pair contributes independently to the denaturation spectrum, irrespective of its nearest neighbours (Felsenfeld & Sandeen, 1962; Fresco *et al.* 1963). This information may be derived from the ratio $\Delta E_{260}/\Delta E_{280}$ since:

$$\Delta E_{260}/\Delta E_{280} = (\delta f_{AU} \Delta\epsilon_{260}^{AU} + \delta f_{GC} \Delta\epsilon_{260}^{GC}) / \delta f_{GC} / \Delta\epsilon_{280}^{GC} \quad (3)$$

where δf_{AU} and δf_{GC} are respectively the mole fractions of A--U and G--C base pairs denaturing over the particular temperature range studied. Eqn. (3) may be rearranged to:

$$\Delta E_{260}/\Delta E_{280} = (\delta f_{AU}/\delta f_{GC}) (\Delta\epsilon_{260}^{AU}/\Delta\epsilon_{280}^{GC}) + \Delta\epsilon_{260}^{GC}/\Delta\epsilon_{280}^{GC} \quad (4)$$

The value of $\Delta\epsilon_{260}^{AU}$ was determined by Fresco *et al.* (1963) whereas $\Delta\epsilon_{260}^{GC}$ and $\Delta\epsilon_{280}^{GC}$ were obtained by placing the denaturation spectrum observed by Fresco *et al.* (1963) for polyGC on a molar basis (see Fig. 1a) by taking into account the fraction of cytosine residues involved in helical secondary structure (Cox, 1965, 1966). Hence by substitution in eqn. (4) and rearranging we have:

$$\delta f_{AU}/\delta f_{GC} = 0.81(\Delta E_{260}/\Delta E_{280}) - 0.46 \quad (5)$$

the relation utilized below.

When a polynucleotide is heated at constant pH in acidic or alkaline solutions its spectrum is affected both by changes in conformation and by changes in the degree of ionization of the base residues, partly owing to the dependence of the dissociation constant (pK) on the absolute temperature:

$$pK = A + B/T \quad (6)$$

where $A = 0.65$ and $B = \Delta H/2.303R$, but principally owing to the effect of denaturation because the pK of a base residue may change by about 2 units as a result of the transition from the double-helical to the amorphous form. The forms of the denaturation spectra at pH values where the base residues ionize were deduced (see Fig. 1) on the basis of the assumption that the effects of ionization and denaturation are additive, and that the difference in the spectrum of the ionized and neutral forms of a base residue is the same as for the corresponding nucleotide. The limiting denaturation spectrum predicted for the transition from the double-helical form, in which the base residues are not

Fig. 1. Denaturation spectra of G--C and A--U base pairs in neutral, acidic and alkaline solutions. (a) Curve I, denaturation spectrum of G--C base pairs in neutral solutions [values of Fresco *et al.* (1963), X I.28; see Cox (1966)]; curve II, calculated denaturation spectrum of G--C base pairs at low pH values [curve I + 0.5 ($\epsilon_{(P)pH1} - \epsilon_{(P)pH}$)CMP]; curve III, calculated spectrum for denaturation at high pH [curve I + 0.5 ($\epsilon_{(P)pH12} - \epsilon_{(P)pH7}$)GMP]. (b) Curve I, denaturation spectrum of A--U base pairs at pH 7 (Fresco *et al.* 1963); curve II, calculated spectrum for denaturation at low pH [curve I + 0.5 ($\epsilon_{(P)pH1} - \epsilon_{(P)pH7}$)AMP]; curve III, calculated denaturation spectrum at high pH [curve I + 0.5 ($\epsilon_{(P)pH12} - \epsilon_{(P)pH7}$)].

ionized, to an amorphous form, in which the ionization of one species of base residue is complete, was calculated by summing the appropriate denaturation spectrum (for 1.0 g. atom of P) with the difference in the spectrum of ionized and neutral forms of the base residue (for 0.5 g. atom of P) concerned (see Fig. 1).

Denaturation in acidic solutions. Thermal denaturation at a constant (acidic) pH affects A--U base pairs according to the equation:

However, since the ionized and neutral forms of adenine have practically the same spectrum, the thermal denaturation spectrum remains effectively the same in acidic as in neutral solutions (Fig. 1b).

The analogous transition that G--C base pairs undergo on thermal denaturation in acidic (0.1 M-sodium phosphate) solutions is:

The left-hand side of eqn. (8) denotes that, in the double-helical form, cytosine residues ionize as a weak acid:

where α is the degree of dissociation, $1-\alpha$ is the fraction of ionized cytosine residues and h is the H^+ concentration; and:

$$\text{p}K = \text{pH} + \log[(1-\alpha)/\alpha] \quad (10)$$

It has been shown (Dove & Davidson, 1962; Cox, 1966) that about 80% of cytosine residues may become protonated without disrupting the double-helical conformation. In the amorphous form the $\text{p}K$ of cytosine residues is 4.70 at 25° (Cox, 1966).

In contrast with adenine residues there is a marked difference in the spectrum of the ionized and neutral forms of cytosine residues, particularly over the range 265–310 m μ . Hence the limiting denaturation spectrum for G--C base pairs (Fig. 1) is considerably enhanced at wavelengths longer than 265 m μ whereas $\Delta\epsilon_{260}$ is hardly affected. The extent to which the limiting spectrum is approached depends on the difference $\delta(1-\alpha)$ in the degree of ionization of cytosine residues as a result of the transition from helical to amorphous forms.

Thus for RNA:

$$\Delta E_{280}/\Delta E_{260} = [\delta f_{\text{GC}} \Delta\epsilon_{280}^{\text{GC}} + \delta(1-\alpha) \Delta\epsilon_{280}^{\text{CH}^+}] / (\delta f_{\text{GC}} \Delta\epsilon_{260}^{\text{GC}} + \delta f_{\text{AU}} \Delta\epsilon_{260}^{\text{AU}}) \quad (11)$$

Now:

$$\delta(1-\alpha) = [\partial(1-\alpha)/\partial f_{\text{GC}}]_T \delta f_{\text{GC}} + [\partial(1-\alpha)/\partial T]_{f_{\text{GC}}} \delta T \quad (12)$$

so that:

$$\Delta E_{280}/\Delta E_{260} = (\delta f_{\text{GC}} \Delta\epsilon_{280}^{\text{GC}} + \{[\partial(1-\alpha)/\partial f_{\text{GC}}]_T \delta f_{\text{GC}} + [\partial(1-\alpha)/\partial T]_{f_{\text{GC}}} \delta T\} \Delta\epsilon_{280}^{\text{CH}^+}) / (\delta f_{\text{GC}} \Delta\epsilon_{260}^{\text{GC}} + \delta f_{\text{AU}} \Delta\epsilon_{260}^{\text{AU}}) \quad (13)$$

where:

$$\Delta\epsilon_{280}^{\text{CH}^+} = \epsilon_{280}(\text{CMP, pH } 1) - \epsilon_{280}(\text{CMP, pH } 7)$$

and:

$$[\partial(1-\alpha)/\partial f_{\text{GC}}]_T = \{[K_{\text{H}}/(h+K_{\text{H}})] - [K_{\text{a}}/(h+K_{\text{a}})]\} \quad (14)$$

and:

$$[\partial(1-\alpha)/\partial T]_{f_{\text{GC}}} = - (B/T^2) e^{2.3[A + (B/T) - \text{pH}]} (1 + e)^{2.3[A + (B/T) - \text{pH}]} \quad (15)$$

where $A = 0.65$ and $B = 1.22 \times 10^3$ for cytosine residues (see eqn. 6). Hence the opposing effects of denaturation and temperature on $1-\alpha$ may be precisely evaluated. For RNA in the amorphous form ($\delta f_{\text{GC}} = 0$) heating will lead to a decrease in $\Delta E_{280}/\Delta E_{260}$ according to eqn. (15). It is evident from eqn. (13) that maximum discrimination

between denaturation of A--U and G--C base pairs is achieved when $[\partial(1-\alpha)/\partial f_{\text{GC}}]_T$ is maximum, i.e. when:

$$\text{pH} = \frac{1}{2}(\text{p}K_{\text{H}} + \text{p}K_{\text{a}}) \quad (16)$$

For cytosine residues at 25° the optimum is pH 3.9 but raising the temperature will lead to a decrease in the optimum pH. A value of about pH 3.9 is convenient for study since the helical secondary structure present at pH 7 persists on titration to about pH 4 at 25°.

Denaturation in alkaline solutions. Thermal denaturation at constant (alkaline) pH leads to the transitions:

and:

Values of $\text{p}K_{\text{H}}$ are not known precisely because, in the double-helical form, ionization of the base residues is completely suppressed even at pH 11.4 (Cox, 1963a). On the basis of previous work a $\text{p}K$ value of 9.70 (cf. Warner & Breslow, 1958) was assigned to both guanine and uracil residues. Thus it is possible, in principle, to bring about the transition from the double-helical form of a polynucleotide in which guanine and uracil residues are not ionized to an amorphous form in which they are completely ionized; the limiting denaturation spectra calculated for A--U and G--C base pairs are given in Fig. 1.

Ionization affects the spectra of both guanine and uracil residues so that the differences in the denaturation spectra are accentuated (Fig. 1), particularly in the region 260–280 m μ . Ionization of uracil residues leads to a decrease in extinction at wavelengths longer than 245 m μ and to an increase in ϵ_{P} at shorter wavelengths, whereas ionization of guanine residues leads to an increase in ϵ_{270} ; ϵ_{230} , ϵ_{260} and ϵ_{278} are unchanged. Empirically it was found that $\Delta E_{270}/\Delta E_{260}$ is a convenient ratio that may be utilized to facilitate the discrimination of the melting ranges of helical segments differing in nucleotide composition.

For A--U base pairs:

$$\Delta E_{270}/\Delta E_{260} = (\delta f_{\text{AU}} \Delta\epsilon_{270}^{\text{AU}} + \delta\alpha_{\text{U}} \Delta\epsilon_{270}^{\text{U}^-}) / (\delta f_{\text{AU}} \Delta\epsilon_{260}^{\text{AU}} + \delta\alpha_{\text{U}} \Delta\epsilon_{260}^{\text{U}^-}) \quad (19)$$

where:

$$\delta\alpha_{\text{U}} = (\partial f_{\text{U}}/\partial f_{\text{AU}})_T \delta f_{\text{AU}} + (\partial\alpha_{\text{U}}/\partial T)_{f_{\text{AU}}} \delta T \quad (20)$$

$\Delta E_{270}/\Delta E_{260}$ remained constant on titration of poly(A+U) with alkali (Cox, 1966).

For G--C base pairs:

$$\Delta E_{270}/\Delta E_{260} = (\delta f_{\text{GC}} \Delta\epsilon_{270}^{\text{GC}} + \delta\alpha_{\text{G}} \Delta\epsilon_{270}^{\text{G}^-}) / (\delta f_{\text{GC}} \Delta\epsilon_{260}^{\text{GC}} + \delta\alpha_{\text{G}} \Delta\epsilon_{260}^{\text{G}^-}) \quad (21)$$

$\Delta\epsilon_{270}^{\text{G}^-}$ is positive and $\Delta\epsilon_{260}^{\text{G}^-}$ is negligible so that in alkaline solutions $\Delta E_{270}/\Delta E_{260}$ is greater (1.75) than the value (1.3) obtained at pH 7 (Cox, 1966); and:

$$\delta\alpha_{\text{G}} = (\partial\alpha_{\text{G}}/\partial f_{\text{GC}})_T \delta f_{\text{GC}} + (\partial\alpha_{\text{G}}/\partial T)_{f_{\text{GC}}} \delta T \quad (22)$$

For RNA:

$$\Delta E_{270}/\Delta E_{260} = (\delta f_{\text{AU}} \Delta\epsilon_{270}^{\text{AU}} + \delta f_{\text{GC}} \Delta\epsilon_{270}^{\text{GC}} + \delta\alpha_{\text{U}} \Delta\epsilon_{270}^{\text{U}^-} + \delta\alpha_{\text{G}} \Delta\epsilon_{270}^{\text{G}^-}) / (\delta f_{\text{AU}} \Delta\epsilon_{260}^{\text{AU}} + \delta f_{\text{GC}} \Delta\epsilon_{260}^{\text{GC}} + \delta\alpha_{\text{U}} \Delta\epsilon_{260}^{\text{U}^-}) \quad (23)$$

It is possible to show by substituting for different values of $\delta f_{AU}/\delta f_{GC}$, when $\alpha_G = \alpha_U = 1$, that $\Delta E_{270}/\Delta E_{260}$ increases as δf_{GC} increases.

For guanine and uracil residues the degree of ionization is the same as the degree of dissociation, so that raising the temperature will increase the degree of ionization of residues in amorphous segments and tend to increase the ratio $\Delta E_{270}/\Delta E_{260}$.

The changes in the spectrum, notably ΔE_{260} , ΔE_{270} and ΔE_{280} , on denaturation analysed above were studied principally for diagnostic purposes. The possibility of analysing the denaturation spectrum to yield δf_{AU} , δf_{GC} , $\delta \alpha_U$, $\delta \alpha_G$ and $\delta \alpha_C$ is examined elsewhere (Cox, 1966).

EXPERIMENTAL

Materials. RNA was isolated from rabbit reticulocytes by a modification (Cox, 1965) of the method of Cox & Arnstein (1963).

RNA was isolated from TYMV by extraction with aqueous phenol. The virus was first purified by differential centrifuging. TYMV (10 mg./ml.) in 0.05 M-KCl was shaken for 1 min. with an equal volume of cold phenol saturated with water. The aqueous layer was extracted a second time with phenol and the RNA was then precipitated with propan-2-ol. The precipitate was redissolved in water containing bentonite (2 mg./ml.) and stored at -20° . The sedimentation pattern of RNA in 0.05 M-KCl revealed that about 60% of the u.v.-absorbing species sedimented as one boundary, the sedimentation coefficient ($S_{20,w}$) being about 30s. The remainder was characterized by $S_{20,w}$ 15–30s.

Ribosomal RNA from *E. coli* was isolated by the method of Littauer & Eisenberg (1959).

The isolation of *E. coli* transfer RNA (which was given by E. M. Martin) was described by Martin, Yegian & Stent (1963).

Yeast RNA was isolated from whole cells by the method of Crestfield, Smith & Allen (1955). The sedimentation pattern showed that degradation to not less than 6s fragments had occurred during isolation.

Crystallizable fragments (about 2.4s; Spencer, Fuller, Wilkins & Brown, 1962) of yeast RNA were given by Dr G. L. Brown.

PolyAGUC, which was given by Dr Grunberg-Manago, was prepared by polymerizing ADP, GDP, UDP and CDP in the respective molar proportions 1:0.5:1:1 with polynucleotide phosphorylase. The product had $S_{20,w}$ about 2.5s although the sedimenting boundary was broad.

Methods. A Unicam SP.700 spectrophotometer fitted with an electrically heated copper block that served as a cell holder (Cox, 1963a) was used for measuring extinctions in the range 220–320 m μ . The stoppered cuvette was weighed before and after heating to ensure that the weight loss due to evaporation was 1% or less. The extinction of the cooled solution was also measured, to verify that the changes in extinction were reversible to within $\pm 1\%$, and in some cases the RNA sample was again heated to check the reproducibility of the dependence of extinction on temperature. Values of E were corrected for changes in concentration on heating due to the expansion of water.

The procedure used for electrometric titrations was as described by Cox & Arnstein (1963). The pH of the solution was measured by means of a micro-electrode covering the range pH 0–14 (Jena Glass combined electrode, 02779

type U; Jenaer Glasswork, Schott und Gen., Mainz, Germany) used in conjunction with an E.I.L. model 39A pH-meter (Electronic Instruments Ltd., Richmond, Surrey).

A Spinco model E ultracentrifuge fitted with u.v. optics was used for all analytical ultracentrifuging. The optical density of the photographic film was measured by means of a double-beam recording micro-densitometer (model E12 Mk IIIB; Joyce-Loebel and Co. Ltd., Newcastle upon Tyne).

RESULTS

Ribosomal RNA from reticulocytes. Ribosomal RNA from rabbit reticulocytes in 0.5 mM-, 10 mM- and 100 mM-sodium phosphate buffer, pH 7.6, was heated to temperatures in the range 25–75°. At each concentration of sodium phosphate the difference in the spectrum of the heated sample and that of a sample maintained at 23–25° was measured. The curves ($E_{(T)} - E_{(25)}$) for RNA in 0.5 mM-phosphate buffer are presented in Fig. 2(a). It is apparent that the difference curves have the same characteristic shape (curve I in Fig. 2b) in the range 25–50° (E_{280}/E_{260} 0.6), whereas the difference curves ($E_{(T)} - E_{(50)}$) in the range 50–75° are bimodal (curve II in Fig. 2b) with maxima at about 247 and 277 m μ and a minimum at 263 m μ (E_{280}/E_{260} 1.2).

The fraction of G + C in helical domains melting to an unfolded form over the range 25–50° was estimated as $50 \pm 2\%$ from the appropriate value of the ratio E_{280}/E_{260} (Fig. 3a) by using eqn. (5) compared with the value of $85 \pm 5\%$ of G + C deduced for domains melting above 50° (Fig. 3b).

The relative values of E_{260} in the range 25–75° given in Fig. 3(c) also reflect the biphasic nature of the transition from helical to unfolded forms. Thus the increase of 15% in E_{260} over the range 25–50° denotes the 'melting' of domains having about

Fig. 2. Denaturation spectra ($E_{(T)} - E_{(25)}$) of reticulocyte ribosomal RNA in 0.5 mM-sodium phosphate buffer, pH 7.6. (a) $T = 30, 35, 40, 45, 50, 55, 60, 65, 70$ and 75° successively. (b) Curve I, $E_{(50)} - E_{(30)}$; curve II, $E_{(75)} - E_{(50)}$. At 25° , $E_{260} = 0.655$.

Fig. 3. Effect of electrolyte concentration on the thermal stability of the secondary structure of reticulocyte ribosomal RNA. (a) $(E_{280}(T) - E_{280(25)}) / (E_{260}(T) - E_{260(25)})$. (b) % of G+C in helical segments denaturing at the temperature indicated, calculated by substituting values of $E_{280}(T_2) - E_{280}(T_1)$ and $E_{260}(T_2) - E_{260}(T_1)$ (where $T_2 > T_1$) in eqn. (5). (c) Dependence of $E_{280}(T) / E_{260}(25)$ on temperature. In each Figure ●, △ and ○ refer respectively to 0.5 mM-, 0.01 M- and 0.1 M-sodium phosphate buffer, pH 7.6.

50% of G+C (T_m 38°). The inflexion noticed over the range 50–55° marks the end of this transition. Above 55° the increase in E_{260} reflects the melting of domains richer in guanine and cytosine residues.

When the concentration of sodium phosphate was increased to 10 mM the difference curves observed were similar to those presented in Fig. 2 although higher temperatures had to be attained to bring about the same increase in extinction. Thus domains of about 50% of G+C 'melted' over the range 25–55° (T_m approx. 44°). Increasing the concentration of sodium phosphate to 100 mM further stabilized secondary structure so that still higher temperatures were required in order to 'melt' out helical domains, e.g. domains of about 57% of G+C 'melted' over the range 35–70°.

Further evidence for the presence of at least two distinct species of helical segments differing in $\delta f_{AU} / \delta f_{GC}$ was obtained by studying the changes in the spectrum of RNA on denaturation in acidic and alkaline solutions. No significant change in melting

temperature was noticed over the range pH 4.5–7.0 but when the solution (0.1 M-sodium phosphate) was brought to pH 4.00 the changes in ΔE_{260} and ΔE_{280} took place over a slightly lower temperature range. Two distinct melting ranges were discerned. The lower-melting species was characterized by a $\Delta E_{280} / \Delta E_{260}$ value of about 0.80, which is higher than that (0.60) observed on denaturation at pH 7 but appreciably lower than value of 1.2 for $(E_{280}(T) - E_{280(66)}) / (E_{260}(T) - E_{260(66)})$ observed over the range 66–86° (Fig. 4c).

When the pH was decreased to pH 3.60 the thermal stability of secondary structure was further diminished and it was easier to distinguish the melting ranges of the two species of helical segments as predicted in the Theoretical section. The dependence of both ΔE_{260} and ΔE_{280} on temperature were clearly biphasic; $\Delta E_{280} / \Delta E_{260}$ was found to be 1.0 in the range 20–53° (compared with 0.6 at pH 7, and 1.8 at pH 4.00), whereas at higher temperatures $\Delta E_{280} / \Delta E_{260}$ was about 1.6 (cf. 1.2 in the range pH 4.0–7.02). Values of $(E_{280}(T) - E_{280(53)}) / (E_{260}(T) - E_{260(53)})$ are given in Fig. 4(b). The denaturation spectrum in the range 25–50° was skewed towards longer wavelengths compared with that observed at pH 7.6 (curve I in Fig. 2b), the maximum being shifted from about 257 mμ at pH 7.6 to 273 mμ at pH 3.6 (curve I Fig. 5b) in accord with transitions (7) and (8). The denaturation spectrum in the range 50–85° more nearly approached the limiting spectrum for the denaturation of G-C base pairs in acidic solutions (eqn. 8); the maximum observed at 245 mμ at pH 7 was no longer apparent but the maximum at about 280 mμ was more prominent (curve II in Fig. 5b).

Decreasing the pH to 3.08 led to a further decrease in the thermal stability of helical secondary structure (Fig. 4a). The less stable species had already 'melted' at 25° at this pH, although the species richer in G-C base pairs persisted. The slight decrease in $\Delta E_{280} / \Delta E_{260}$, i.e. $(E_{280}(T) - E_{280(32)}) / (E_{260}(T) - E_{260(32)})$, relative to pH 3.60 is attributed to the decrease in the term $(\partial \alpha / \partial f_{GC})_T$ (see eqn. 12).

When the solution was brought to pH 9.80 the secondary structure decreased slightly in thermal stability. The dependence of ΔE_{260} and ΔE_{270} on temperature was found to be biphasic (Fig. 4e) and each phase was characterized by a particular denaturation spectrum (Fig. 6). Over the range 25–50° the difference spectrum shown by curve I in Fig. 6(b) was observed; the maximum was found at 266 mμ and $\Delta E_{270} / \Delta E_{260}$ was about the same as at pH 7.

Curve II in Fig. 6(b) was typical of denaturation at higher temperatures; only one maximum, at 272 mμ, was noted, in contrast with maxima at 245 and 280 mμ at pH 7. Thus both species of

Fig. 4. Thermal denaturation of rabbit reticulocyte ribosomal RNA in 0.1 M-sodium phosphate buffers of different pH values. (a) At 19.9°, $E_{260} = 0.352$ and $E_{280} = 0.218$; for the lower-melting range $T_1 = 19.9^\circ$, otherwise $T_1 = 31.3^\circ$. (b) At 20.9°, $E_{260} = 0.345$ and $E_{280} = 0.187$; $T_1 = 20.9^\circ$ for the lower-melting range, otherwise $T_1 = 52.8^\circ$. (c) At 17.9°, $E_{260} = 0.343$ and $E_{280} = 0.172$; $T_1 = 23.7^\circ$ over the range 23.7–66.6°; otherwise $T_1 = 66.6^\circ$. (d) At 25°, $E_{260} = 0.71$ and $E_{280} = 0.31$; $T_1 = 25^\circ$ for the lower-melting range, otherwise $T_1 = 70^\circ$. (e) At 25°, $E_{260} = 0.876$ and $E_{270} = 0.669$; $T_1 = 25^\circ$ for the lower-melting range, otherwise $T_1 = 45^\circ$. (f) At 25°, $E_{260} = 0.876$ and $E_{270} = 0.670$; $T_1 = 25^\circ$ for the lower-melting range, otherwise $T_1 = 45^\circ$.

Fig. 5. Denaturation spectrum ($E(T) - E_{(25)}$) of reticulocyte ribosomal RNA in 0.1 M-sodium phosphate buffer, pH 3.6. (a) $T = 25, 30, 35, 40, 45, 50, 55, 60, 65, 70$ and 75° successively. (b) Curve I, $E_{(40)} - E_{(25)}$; curve II, $E_{(75)} - E_{(50)}$. At 25° , $E_{280} = 0.850$.

Fig. 6. Denaturation spectrum ($E(T) - E_{(25)}$) of reticulocyte ribosomal RNA in 0.1 M-sodium phosphate buffer, pH 9.80. (a) $T = 25, 30, 35, 40, 45, 50, 55, 60, 65, 70$ and 75° successively. (b) Curve I, $E_{(40)} - E_{(30)}$; curve II, $E_{(75)} - E_{(40)}$. At 25° , $E_{280} = 0.876$.

denaturation spectra are consistent with the denaturation of complementary base pairs in alkaline solutions (eqns. 17 and 18).

In more alkaline solutions, pH 10.32, secondary structure was less stable than at pH 9.80. The dependence of ΔE_{260} and ΔE_{270} on temperature was again biphasic (Fig. 4f). The difference spectra are given in Fig. 7: curve I in Fig. 7(b), which was characteristic of denaturation over the range

25–45°, was found to have a maximum at about 263 mμ ($\Delta E_{270}/\Delta E_{260} 1.0$), and curve II in Fig. 7(b) had the same features as curve II in Fig. 6(b), although the $\Delta E_{270}/\Delta E_{260}$ value of 1.7 was somewhat higher. The difference spectra observed on heating both acidic and alkaline solutions of RNA are consistent with the denaturation of complementary base pairs.

Fig. 7. Denaturation spectrum ($E_{(T)} - E_{(25)}$) of reticulocyte ribosomal RNA in 0.1 M-sodium phosphate buffer, pH 10.32. (a) $T = 25, 30, 35, 40, 45, 50, 55, 60, 65, 70$ and 75° successively. (b) Curve I, $E_{(35)} - E_{(25)}$; curve II, $E_{(75)} - E_{(45)}$. At 25° , $E_{260} = 0.876$.

Fig. 8. Denaturation spectrum ($E_{(T)} - E_{(25)}$) of *E. coli* RNA in 5 mm-sodium phosphate buffer, pH 7.00. (a) $T = 25, 30, 35, 40, 45, 50, 55, 60, 65, 70$ and 75° successively. (b) Curve I, $E_{(50)} - E_{(30)}$; curve II, $E_{(75)} - E_{(50)}$. At 25° , $E_{260} = 0.876$.

Unfractionated RNA from whole ribosomes was used in these experiments so that the results observed reflect principally the properties of the faster-sedimenting component. However, preliminary experiments showed that both the 16S and 30S components of RNA isolated from the fractionated ribosomal sub-units (prepared by titration with EDTA) (Arnstein, Cox, Gould & Potter, 1965) after fractionation had similar denaturation spectra in 0.5 mM-sodium phosphate buffer, pH 7. The melting ranges of the two species of helical segments were not identical and probably reflect the difference in the nucleotide composition of the two components (H. Gould, H. R. V. Arnstein & R. A. Cox, unpublished work; cf. Montagnier & Bellamy, 1964).

Fig. 9. Effect of electrolyte concentration on the thermal stability of the secondary structure of *E. coli* RNA. (a) $(E_{280}(T) - E_{280}(25)) / (E_{260}(T) - E_{260}(25))$. (b) % of G+C in helical segments denaturing at the temperature indicated, calculated according to the procedure given in the legend to Fig. 3. (c) Dependence of $E_{260}(T) / E_{260}(25)$ on temperature. In each Figure ●, Δ and ○ refer respectively to 0.5 mM-, 0.01 M- and 0.10 M-sodium phosphate buffer, pH 7.0.

Ribosomal RNA from E. coli. The experiments described above were repeated with RNA from *E. coli* ribosomes. The difference spectra obtained in 0.5 mM-sodium phosphate buffer, pH 7.0, are given in Fig. 8. Two distinct species could be discerned. In the range 30–50° the difference spectra ($E_{(T)} - E_{(25)}$) were characterized (curve I in Fig. 8b) by a maximum at about 267 mμ and a $\Delta E_{280} / \Delta E_{260}$ value of 0.55. In contrast, above 50° values of E in the range 240–280 mμ were similar to one another ($\Delta E_{280} / \Delta E_{260} 0.8$).

Of the base pairs in helical segments denaturing over the range 30–50° the fraction of G+C was estimated as $50 \pm 4\%$ from eqn. (5), whereas helical segments melting above 55° were richer ($67 \pm 4\%$) in G+C.

Fig. 10. Thermal denaturation of *E. coli* RNA in 0.1M-sodium phosphate buffers at different pH values. (a) At 23.5°, $E_{260} = 0.590$ and $E_{280} = 0.304$; for the lower-melting phase $T_1 = 23.5^\circ$, otherwise $T_1 = 51.7^\circ$. (b) At 25°, $E_{260} = 0.581$ and $E_{280} = 0.289$; for the lower-melting phase $T_1 = 25^\circ$, otherwise $T_1 = 45^\circ$. (c) At 21°, $E_{260} = 0.495$ and $E_{280} = 0.233$; for the lower-melting phase $T_1 = 21^\circ$, otherwise $T_1 = 54^\circ$. (d) At 25°, $E_{260} = 0.892$ and $E_{280} = 0.395$; for the lower-melting phase $T_1 = 25^\circ$, otherwise $T_1 = 60^\circ$. (e) At 25°, $E_{260} = 0.907$ and $E_{270} = 0.687$; $T_1 = 25^\circ$ or 45° . (f) At 25°, $E_{260} = 0.927$ and $E_{270} = 0.720$; $T_1 = 30^\circ$ except over the range 25–30° when $T_1 = 25^\circ$.

The relative increase in ΔE_{260} found over the range 25–75° is given in Fig. 9(c). The increase of about 17% (T_m approx. 40°) over the range 25–50° reflects the denaturation of helical segments of about 50% of G + C. The increase in E_{260} above 55° is attributed to the 'melting' of domains richer in G + C.

Increasing the concentration of sodium phosphate to 10mM did not affect the temperature range over which denaturation took place (Fig. 9c), probably because of traces of bivalent cations in the stock RNA solution. However, increasing the electrolyte concentration to 0.1M led to an increase in the stability of helical secondary structure. The difference curves obtained on denaturation were the same as those presented in Fig. 8 but higher temperatures were required to bring about the same transitions, e.g. segments of about 50% of G + C denatured over the range 30–60° (cf. 24–50° in 5mM solutions).

The denaturation spectrum was hardly affected by the pH of the solution in the range pH 4.5–8.0. However, at pH 4.00 denaturation took place at

slightly lower temperatures. Moreover, the dependence of ΔE_{260} and ΔE_{280} on temperature were both clearly biphasic (Fig. 10c) and an inflexion was noted at 55°. Over the range 25–55° the denaturation spectrum was skewed towards longer wavelengths (curve I in Fig. 11b) so that a $\Delta E_{280}/\Delta E_{260}$ value of 0.80 was found (cf. 0.55 at pH 7), and the melting range was slightly displaced towards lower temperatures. Above 55° the denaturation spectrum was further skewed towards longer wavelengths ($\Delta E_{280}/\Delta E_{260}$ 1.0).

At pH 3.60 the melting range was further displaced so that part of the transition from helical to amorphous forms had already taken place at 25° (Fig. 10b). Increasing the temperature from 25 to 87° led to differences in the spectrum consistent with the denaturation of two distinct species of helical segments as reported above; the species melting to an amorphous form below 45° was characterized by $\Delta E_{280}/\Delta E_{260}$ 0.85, and above 45° $\Delta E_{280}/\Delta E_{260}$ was higher and decreased from 1.4 at 50° to 1.0 at 75° (see eqn. 15).

In more acidic solutions (pH 3.11) denaturation

Fig. 11. Denaturation spectra of *E. coli* RNA in 0.1M-sodium phosphate buffer, pH 4.06. (a) $T = 25, 30, 35, 40, 45, 50, 55, 60, 65, 70$ and 75° successively. (b) Curve I, $E_{(40)} - E_{(30)}$; curve II, $E_{(45)} - E_{(30)}$. (c) Curve I, $E_{(65)} - E_{(55)}$; curve II, $E_{(75)} - E_{(50)}$. At 25° , $E_{260} = 0.900$.

Fig. 13. Denaturation spectra of *E. coli* RNA in 0.1M-sodium phosphate buffer, pH 10.30. (a) $T = 25, 30, 35, 40, 45, 50, 55, 60, 65, 70$ and 75° (the differences in curves for 65 and 70° were identical). (b) Curve I, $E_{(30)} - E_{(25)}$; curve II, $E_{(70)} - E_{(30)}$. At 25° , $E_{260} = 0.927$ and $E_{270} = 0.720$.

Fig. 12. Denaturation spectra of *E. coli* RNA in 0.1M-sodium phosphate buffer, pH 9.80. (a) $T = 25, 30, 35, 40, 45, 50, 55, 60, 65, 70$ and 75° successively. (b) Curve I, $E_{(35)} - E_{(25)}$; curve II, $E_{(65)} - E_{(45)}$. At 25° , $E_{260} = 0.907$ and $E_{270} = 0.687$.

Fig. 14. Titration of reticulocyte ribosomal RNA in 0.1M-NaCl at 25° . Curve I, ●, titration with acid from pH 7. Curve II, ○, titration with alkali from pH 3.0. Curve III, RNA fragments: ●, titration with acid from pH 7; ○, titration with alkali from pH 3 (○, coincident ● and ○).

took place at still lower temperatures and only the species richer in G + C persisted at 25° ($\Delta E_{280}/\Delta E_{260}$ 1.0). Above 50° a $\Delta E_{280}/\Delta E_{260}$ value of 0.5 was observed (Fig. 10a). Previously, evidence has been presented (Cox & Littauer, 1963) suggesting that at this pH the changes in the spectrum noticed above 55° are due to the denaturation of acid-stable secondary structure.

When the pH was raised from 7 to 9.80 denaturation was observed at lower temperatures (Figs. 10 and 12). Denaturation in the range 25 – 50° was characterized by the difference spectrum shown by

curve I in Fig. 12(b), the value of $\Delta E_{270}/\Delta E_{260}$ being 1.05. The difference spectrum shown by curve II in Fig. 11(b) was observed on denaturation above 50° and has a well-defined maximum at about $267m\mu$, as would be expected on denaturation of G + C-rich segments: the value of 1.2 was found for $\Delta E_{270}/\Delta E_{260}$.

At pH 10.30 the thermal stability of secondary structure was decreased still further, and for helical segments of about 50% of G + C part of the transition to an amorphous form had already taken place at 25° and was complete at 35° (Fig. 10f). The

difference spectra obtained are given in Fig. 13. The difference in the spectrum brought about by increasing the temperature from 25 to 30° (curve I in Fig. 12*b*) is comparable with curve I in Fig. 11*b*). Increasing the temperature over the range 30–78° led to a more pronounced increase in E_{270} ($\Delta E_{270}/\Delta E_{280}$ 1.3; cf. 1.2 at pH 9.80), as shown by curve II in Fig. 13*b*).

It is concluded that the changes in the spectrum of *E. coli* ribosomal RNA on denaturation, like those of reticulocyte ribosomal RNA, are entirely consistent with the denaturation of complementary base pairs. Moreover, there appear to be two principal species of helical segments, one having a nucleotide composition of about 50% of G + C, the other having about 67% of G + C.

Preliminary experiments with RNA from yeast showed that its secondary structure was essentially similar to that of *E. coli* RNA and reticulocyte

ribosomal RNA although the nucleotide composition of the low- and high-melting phases were similar, about 55% of G + C and 60% of G + C respectively. The melting range was the same for both the crystallizable fragments of mol.wt. 14800 (W. B. Gratzer, unpublished work; quoted by Spencer & Poole, 1965) and higher-molecular-weight RNA, indicating that decreasing the mol.wt. to about 15000 did not appreciably affect helical secondary structure.

Electrometric titration studies. Reticulocyte ribosomal RNA (4mg./ml.) in 0.1M-sodium chloride was treated at 25° with hydrochloric acid from pH 7 to pH 3.8 (curve I in Fig. 14), and the same curve was followed on titration with alkali from pH 3.8 to 7.0. However, after titration to more acidic pH values, e.g. pH 3.0, a different curve (II in Fig. 14) was followed on titration with alkali (cf. titration of rat-liver ribosomal RNA; Cox, 1963*a*). Curve I

Fig. 15. Titration of crystallizable fragments (2.4s) of yeast RNA in 0.1M-NaCl at 25°.

in Fig. 14 was subsequently followed on titration with acid from pH 7, and curves I and II in Fig. 14 were accurately reproduced on successive titration cycles.

The titration properties of reticulocyte ribosomal RNA degraded at pH 13 by the method of Spencer & Poole (1965) were found to be essentially independent of molecular size provided that the fragments in 0.1 M-sodium phosphate buffer, pH 7, were characterized by $S_{20,w}$ values greater than about 2s. However, 1s fragments (curve III in Fig. 14) titrated over a higher pH range, as would be expected if secondary structure were absent. Moreover, the titration curve was reversible.

High-molecular-weight RNA from yeast as well as crystallizable (2.4s) fragments were also titrated. As reported above for reticulocyte RNA, hysteresis was observed over the range pH 3–7. Titration with acid from pH 7 to pH 4.0 followed the same course as titration with alkali from pH 4.0 to pH 7.0 (curve I in Fig. 15). A different curve (II in Fig. 15) was followed on titration with alkali from pH 3.6. In this case, too, curves I and II, which were the same for both crystallizable fragments and high-molecular-weight RNA, were reproduced on repeated titration cycles.

Hysteresis was also noted on titration of TYMV RNA in 0.1 M-sodium chloride at 25° (Fig. 16a) but the difference in equiv. of acid bound/4g.atoms of P at a particular pH value along curves I and II was never more than 0.06 compared with 0.12 for yeast RNA and 0.16 for reticulocyte RNA.

The dependence of E_{260} on temperature at different pH values is given in Fig. 16(b). It is evident that the stability of the secondary structure of TYMV RNA decreased as the pH decreased. However, the persistence of a small increment in E_{260} over the range 25–50° at pH 2.80 is attributed to the denaturation of secondary structure that is stable in acidic but not in neutral solutions at 25° (cf. *E. coli* RNA; Cox & Littauer, 1963).

Transfer RNA from *E. coli* that was titrated over the range pH 3.0–7.0 had the same general features (Fig. 17) as TYMV RNA (Fig. 16). Although hysteresis was again observed, the difference in the equivalents of acid bound/4g.atoms of P was never more than 0.05; this difference could be accounted for by the presence of about 25% of ribosomal RNA fragments in the preparation.

Fig. 16. (a) Effect of pH and temperature on E_{260} of TYMV RNA in 0.1 M-sodium phosphate buffer: ○, pH 2.80; □, pH 3.09; △, pH 3.60; ●, pH 4.01; ■, pH 4.42–7.0. (b) pH-titration curve for TYMV RNA in 0.1 M-NaCl at 25°.

Fig. 17. pH-titration curve for *E. coli* transfer RNA in 0.1 M-NaCl at 25°.

Fig. 18. Effect of pH and temperature on E_{260} of polyAGUC in 0.1 M-sodium phosphate buffer: ○, pH 2.80; △, pH 2.98; ●, pH 3.60; □, pH 4.01–7.0.

Insufficient polyAGUC was available for titration studies. However, it is possible to correlate hysteresis with the presence of acid-stable secondary

structure as revealed by the dependence of E_{260} on temperature at different pH values. When poly-AGUC was studied in this way (Fig. 18) it was found

Fig. 19. Phase diagram for reticulocyte ribosomal RNA. (a) Dependence of T_m on pH (in 0.1 M-sodium phosphate buffer). The ordinate $\Delta\epsilon_{260}/\Delta T$ indicates the relative proportion of base pairs in helical segments denaturing at a particular temperature. The area under the curve $\Delta\epsilon_{260}/\Delta T$ against temperature is equal to the total increase in E_{260} . ■ denotes helical segments of about 50% of G + C; ▨ indicates helical segments of about 85% of G + C; ▩ denotes the melting profile of acid-stable secondary structure obtained by heating RNA in 0.01 M-sodium phosphate buffer, pH 3.0; ▧ indicates overlapping ▨ and ▩. (b) $\Delta\epsilon_{260}/\Delta pH$ derived from the data of Cox & Arnstein (1963). (c) Spectrophotometric titration data (Cox & Arnstein, 1963).

that decreasing the pH led to the elimination of secondary structure and no evidence for the presence of stable secondary structure in acidic solutions was obtained.

DISCUSSION

Nature of base-pairing in ribosomal RNA. There is increasing evidence that the hyperchromism of

polynucleotides, which is due to the tendency of the base residues to stack one upon another, may be manifest when it is unlikely that double-helical structures are formed (e.g. Van Holde, Brahms & Michelson, 1965). Although a change in extinction is observed when a double-helical segment melts to an unfolded form it does not follow that an increase in extinction obtained by raising the temperature

Fig. 20. Phase diagram for *E. coli* ribosomal RNA. (a) Dependence of T_m on pH (in 0.1 M-sodium phosphate buffer). The plot of $\Delta \epsilon_{260} / \Delta T$ has the same significance as in Fig. 19(a). ■ and ▨ refer respectively to helical segments of about 50% and 67% of G + C. (b) $\Delta \epsilon_{260} / \Delta pH$, derived from the data of Cox & Littauer (1963). (c) Spectrophotometric titration data (Cox, 1961).

Fig. 21. Phase diagram for *E. coli* RNA in 0.1M-NaCl. P-Q indicates the temperature range over which E_{260} increases at pH7, the horizontal markers indicating the temperature interval over which E_{260} increased by 1%. ○-○ denotes the pH range over which, on titration from pH7, the forward (cf. curve I in Fig. 14) and back titration (cf. curve II in Fig. 14) curves are generated: e.g. at 25° the base residues ionize reversibly on titration from pH7 to pH4 and back to pH7.0, whereas after titration to more acidic pH values the forward and back titration curves are no longer identical, the difference being maximum at pH3.6. Hence the zone AOB-A'O'B' delineates the stability of the secondary structure that has to be disrupted to allow the formation of acid-stable structure. ●-● indicates the pH range, on titration with alkali from pH3.0, over which the difference between the forward (in this case cf. curve II in Fig. 14) and back (cf. curve I in Fig. 14) titration curves is generated: e.g. at 25° the base residues ionize reversibly on titration from pH3.0 to pH4.1 and back to pH3.0, whereas after titration to pH values in the range pH4.1-4.7 a curve intermediate between curves I and II was followed on back titration with acid. A further change in the difference between forward and back titration curves was noticed when the pH was brought from pH3 to in the range pH5.8-6.3: when at pH6.3 (or higher) curve I was followed on subsequent titration with acid. The zone FG-F'G' delineates the stability of secondary structure that has to be disrupted to allow the original secondary structure that is stable at pH7 to re-form. The curve R-S indicates the increase in E_{260} noted on heating *E. coli* RNA in 1 mM-NaCl, pH3.0. The horizontal marker indicates an increase of 1% in E_{260} .

Table 1. Nucleotide composition of helical and amorphous segments of ribosomal RNA

Values in parentheses were derived from spectrophotometric titrations at 25° (cf. Cox, 1961). The values for reticulocyte RNA were derived from the spectrophotometric measurements of Cox & Arnstein (1963).

	Nucleotide composition (molar proportions)					
	Reticulocyte RNA			<i>E. coli</i> RNA		
	Total	Helical	Amorphous	Total	Helical	Amorphous
A	0.7	0.48 (—)	0.22 (—)	1.08	0.62 (0.58)	0.46 (0.50)
G	1.44	0.92 (1.3)	0.50 (0.14)	1.18	0.78 (0.8)	0.40 (0.35)
U	0.72	0.48 (0.42)	0.24 (0.3)	0.82	0.62 (0.53)	0.20 (0.35)
C	1.12	0.92 (1.12)	0.20 (0.1)	0.92	0.78 (0.72)	0.14 (0.2)
% of G+C ...	—	66	—	—	56	—

from the pH-titration curve (Cox, 1961; Cox & Littauer, 1963).

The spectrophotometric titration data also support the conclusion that there are two distinct species of helical segments that differ in nucleotide composition (cf. Cox, 1961). When reticulocyte RNA was titrated with acid at 25° secondary structure was unchanged over the range pH 4.0–7.0; the titration curve (curve I in Fig. 14) was reversible, and E_{280}/E_{260} increased over this range (Fig. 19) owing solely to the ionization of cytosine residues in amorphous segments. When the pH was further decreased, E_{280}/E_{260} increased, partly owing to the ionization of residues in helical segments (pK 3.1) and partly owing to an increase in the proportion of residues in amorphous segments as a result of denaturation. At pH 3.0, however, helical segments of 50% of G + C were denatured, the titration curve was no longer reversible (curve II in Fig. 14), and the secondary structure at this pH, indicated by the observation that not all cytosine residues were ionized, was identified from the denaturation spectrum as the segments richer in G + C.

Titration with alkali, at 25°, led first to the ionization of guanine and uracil residues in amorphous segments over the range pH 8.0–10.0; the proportion of uracil residues titrating over this range is greater than that of guanine residues. Continuing the titration to pH 11.0 led to the ionization of most of the uracil residues and to a smaller fraction of guanine residues as helical segments of 50% of G + C became denatured. Increasing the pH still further led to the ionization of the remaining guanine and uracil residues as helical segments richer in G + C denatured.

The same correspondence between the pH-titration properties and the denaturation spectra was observed for *E. coli* RNA (Fig. 20). Previously it was noted (Cox & Littauer, 1963) that titration from pH 7.0 to pH 4.5 at 25° led to no substantial change in E_{260} , indicating that secondary structure

was unchanged over this range; the titration curve was reversible (cf. curve I in Fig. 14), and the increase in E_{280}/E_{260} was attributed to the ionization of cytosine residues in amorphous segments. Decreasing the pH further led to an increase in E_{260} with an inflexion at pH 3.3, and an increase in E_{280}/E_{260} to an extent consistent with the denaturation of helical segments poorer in G + C; a different integral titration curve (cf. curve II in Fig. 14) was followed on titration from pH 3.3. Helical segments richer in G + C were completely denatured at about pH 2.7.

Titration with alkali led first to the titration of guanine and uracil residues in amorphous segments over the range pH 8.0–9.8 and then to denaturation. The difference in the titration ranges of guanine and uracil residues was not as marked as for reticulocyte RNA, in accord with the inference that the difference in the nucleotide composition of the two species of helical segment is less for *E. coli* RNA.

The overall nucleotide composition of the helical segments ($f_{GC(\text{total})}$) is due to fractions x and $1-x$ respectively of guanine plus cytosine residues in helical segments of low ($f_{GC(I)}$) and high ($f_{GC(II)}$) G + C content so that:

$$f_{GC(\text{total})} = xf_{GC(I)} + (1-x)f_{GC(II)} \quad (24)$$

In both *E. coli* RNA and reticulocyte RNA guanine and cytosine residues appear to be equally distributed among both species of helical segment: the species poorer in G + C appears to be shorter in chain length (see below), and probably occurs more frequently.

Chain length of helical segments. The double-helical segments of ribosomal RNA denature at lower temperature range than the highly polymerized RNA of the same average nucleotide composition, e.g. helical segments of about 50% of G + C 'melt' over the range 40–70° whereas the replicative form of encephalomyocarditis-virus RNA, which is believed to be double-stranded, has

a similar nucleotide composition but 'melts' at 96° (solvent, 0.15M-sodium chloride-0.015M-sodium citrate; Montagnier & Sanders, 1963). Studies with model compounds have shown that when N , the number of base pairs per helix, is small (about 20 or less) the melting temperature, T_m , depends on chain length. For ribosomal RNA N is probably also small.

OligoA of different chain lengths interacts with polyU forming double-helical structures [cf. poly(A+U)] having a well-defined melting range (Doty, 1961), and T_m and N are related by the equation (Lipsett, Heppel & Bradley, 1961):

$$T_m = 72 - (169/N) \quad (25)$$

and Lipsett (1964) found for poly(G+C) obtained by interaction of oligoG with polyC that:

$$T_m + 103 - (174/N) \quad (26)$$

The intercept but not the slope of eqns. (25) and (26) depends on the nucleotide composition of the double helix and in the general case:

$$T_m = T_{m(\infty)} - (172/N) \quad (27)$$

where $T_{m(\infty)}$ refers to the limiting value when N is large. Eqn. (27) was used to estimate N for the helical segments of reticulocyte ribosomal RNA and a range of 4–8 base pairs was calculated for segments of about 50% of G+C melting at 40–70°, and of 5–17 base pairs for the species of about 85% of G+C melting over the range 75–95° [in this case $T_{m(\infty)}$ was obtained by interpolation between the values of $T_{m(\infty)}$ given for poly(A+U) and poly(G+C) in eqns. (25) and (26)]. The helical segments of *E. coli* RNA were estimated to be within the range 3–8 base pairs, the average length of species of 50% of G+C being about 4 base pairs compared with an average of 6 base pairs calculated for the species richer in G+C. These estimates take no account of the possibility that T_m depends on nucleotide sequence as well as nucleotide composition, or that the helical segments may be imperfect (Fresco, Alberts & Doty, 1960; Bautz & Bautz, 1964).

The observation that 2s fragments (45 nucleotides long; Spencer *et al.* 1962) obtained by hydrolysing ribosomal RNA in the amorphous form have essentially the same helical secondary structure as the intact molecule (Figs. 14 and 15) shows that helical segments are shorter than the fragments, i.e. N is less than 22 base pairs. Once the average size of the fragments is less than the average size of helical segments, the original secondary structure will not re-form (e.g. curve III in Fig. 14, which characterizes 1s fragments) and a lower limit of helical size may be obtained, in this case about 6 base pairs.

Nucleotide sequence and hysteresis. The total

hyperchromicity at 260m μ noted for *E. coli* transfer RNA, TYMV RNA and ribosomal RNA (Figs. 3, 9 and 16) is similar, indicating that the amount of secondary structure is the same. However, the hysteresis observed on titration over the range pH 3–7 is greatest for ribosomal RNA irrespective of its nucleotide composition. This difference in properties is attributed to the presence in ribosomal RNA of a number of occasional clusters of adenine (or possibly cytosine) residues. A hypothetical polynucleotide chain that may form secondary structure having the average properties deduced for

Fig. 22. Possible changes in the secondary structure of a polynucleotide having the same average nucleotide composition as reticulocyte ribosomal RNA due to titration from pH 7 to pH 3 and back to pH 7. (a) Conformation stable at pH 7. (b) Ionized form of (a) (see eqns. 7 and 8). (c) Helical segments poorer in G+C 'denature' on decreasing the pH below about 3.6, allowing the formation of acid-stable double-helical segments where the chains run parallel to one another (cf. Rich, Davies, Crick & Watson, 1961). (d) Apart from changes in the degree of ionization (c) persists until the pH is increased beyond about 5.6, when the acid-stable segments 'melt', so permitting (a) to re-form.

reticulocyte ribosomal RNA at pH 7 is represented in Fig. 22 (a). At 25°, over the range pH 4–7, secondary structure remains stable although adenine and cytosine residues ionize (Fig. 22b) according to eqns. (7) and (8). As the pH is further decreased the helical segments richer in A+U denature, permitting the formation of oligoA-like segments (Fig. 22c) that are stable in acidic but not in neutral or alkaline solutions (cf. Steiner & Beers, 1959); the polynucleotide chains run parallel to one another in this form (cf. Rich, Davies, Crick & Watson, 1961), and the pK of adenine residues is higher than that of AMP (Steiner & Beers, 1959). Thermodynamic equilibrium between the forms depicted in Figs. 22(b) and 22(c) is not attained (Cox, 1963a), so that subsequently raising the pH, e.g. to 4.5, does not alter the conformation, the original structure (Fig. 22b) cannot re-form, and the number of protons bound remains higher than at a comparable pH in forward titration (Fig. 22b). Once the pH is increased to a value where oligoA segments 'melt', the original structure re-forms, and the cycle may be repeated. There is no evidence that cytosine residues also tend to be found in clusters, although such clusters could also form oligoC segments (cf. polyC; Hartman & Rich, 1966) that are stable in acidic solutions and so contribute to hysteresis. *E. coli* transfer RNA and TYMV RNA appear to have fewer clusters of adenine or cytosine residues. Thus the hypothetical polynucleotide (Fig. 22a) appears to have the same denaturation and acid-base properties as reticulocyte ribosomal RNA.

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