A Spectrophotometric Study of the Secondary Structure of Ribonucleic Acid Based on a Method for Diminishing Single-Stranded Base-'Stacking' without Affecting Multi-helical Structures

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1. On the basis of studies with model compounds it was concluded that in 8M-urea–M-potassium chloride (or 4M-guanidinium chloride) in 0·01M-potassium phosphate buffer, pH 7·0, multi-helical structures have about the same stability as in 0·1M-potassium phosphate buffer, pH 7·0, whereas the tendency of base residues to 'stack' along a single polynucleotide chain is much decreased. 2. Base-pairing was eliminated whereas base-'stacking' persisted after RNA in 1% formaldehyde–0·1M-potassium phosphate buffer, pH 7·0, was heated to 95°. 3. From a study of the thermal denaturation of unfractionated transfer RNA from *Escherichia coli* and of RNA from the fractionated sub-units of rabbit reticulocyte ribosomes in 8M-urea–M-potassium chloride (or 4M-guanidinium chloride) in 0·01M-potassium phosphate buffer, pH 7·0, it was inferred that 'stacked' residues may account for up to 25% of the increase in E_{260} found on heating RNA in solvents such as 0·1M-potassium phosphate buffer, pH 7·0. 4. Changes in the spectrum with temperature were analysed on the basis of the assumptions that (*a*) the polynucleotide chain is amorphous on denaturation (which is probable in 8M-urea–M-potassium chloride–0·01M-potassium phosphate buffer, pH 7·0) and that (*b*) the polynucleotide chain adopts a single-stranded 'stacked' conformation on denaturation (which is probable when ordinary solvents such as 0·1M-potassium phosphate buffer, pH 7·0, are used).

The elucidation of the primary sequence of several t-RNA* molecules (Holley *et al.* 1965; Zachau, Dütting & Feldman, 1966; Madison, Everett & Kung, 1966; Bayev *et al.* 1966) has revealed the inadequacy of methods for studying their secondary structure. The main difficulties arise because the changes in the secondary structure of t-RNA (and other single-stranded polynucleotides) appear to take place over the same wide temperature range as single-stranded structures. The difference in the spectrum of t-RNA below 300mμ observed on heating to 95° (the denaturation spectrum) has been analysed in terms of the base pairs denaturing over a particular range (Felsenfeld & Cantoni, 1964; Fresco, Klotz & Richards, 1963). It has now been established that base-'stacking' along a single polynucleotide chain is sufficient to give rise to hypochromism (Fasman, Lindblow & Grossman, 1964; Holcomb & Tinoco, 1965; Van Holde, Brahms & Michelson, 1965; Brahms, Michelson & Van Holde, 1966; Leng & Felsenfeld, 1966; Vournakis, Scheraga, Rushizky & Sober, 1966; Poland, Vournakis & Scheraga, 1966). Analysis is further complicated because the denaturation spectra of both oligoA and poly(A + U) have the same form, and because the denaturation spectra of both oligoG and oligoC have the main features of the various difference spectra reported for polyGC (Fresco *et al.* 1963; Pochon & Michelson, 1965). The denaturation spectrum of a mixture of approximately equal amounts of oligoA and oligoG was found (Cox & Kanagalingam, 1967) to be hardly distinguishable from the denaturation spectrum for 40% of G--C and 60% of A--U base pairs given by Fresco *et al.* (1963).

It is possible to provide evidence for the presence of double-helical structures by studying the denaturation spectrum of a polynucleotide at

* Abbreviations: t-RNA, transfer RNA; A, C, G and U, adenine, cytosine, guanine and uracil respectively; A--U etc., base residues indicated paired in authentic double-helical structure in which the base residues are located on separate segments of either the same or different polynucleotide chains; oligoA etc., polymer 6–8 residues in length of AMP etc.; polyA etc., polymer of AMP etc.; poly(A + U), double-helical complex formed between polyA and polyU; poly(A + 2U), triple-stranded complex formed between polyA and polyU (AMP/UMP ratio 1:2); polyAU etc., co-polymer of AMP and UMP etc.

different pH values and ionic strengths (Cox, 1966*a*). These methods exploit the knowledge that the pH range over which a base residue ionizes is substantially different for double-helical and single-stranded conformations (Cox, 1966*b*), and that the thermal stability of double-helical structures is dependent on the concentration of added electrolyte (Dove & Davidson, 1962; Schildkraut & Lifson, 1965) to a greater extent than that found for single-stranded 'stacked' structures (Cantor, Jaskunas & Tinoco, 1966; Cox & Kanagalingam, 1967). Repulsion between negatively charged phosphate residues located on opposite strands of the double helix appears to account for the greater effect of electrolyte concentration on the stability of double-stranded compared with single-stranded structures (Schildkraut & Lifson, 1965).

Quantitative analysis of the denaturation spectrum is still further complicated if, as appears probable, a double-helical segment reverts to a single-stranded 'stacked' structure on denaturation. t-RNA may exist in a single-stranded 'stacked' structure (Fasman, Lindblow & Seaman, 1965) after treatment with formaldehyde. Partial degradation of reticulocyte ribosomal RNA leads to small fragments that have properties consistent with single-stranded 'stacked' structures (Cox & Kanagalingam, 1967). The copolymer polyAU reverts also to a form comparable with the alkaline form of polyA on denaturation (Cox, 1963*b*), and polyA is now known to be a single-stranded 'stacked' structure (e.g. Brahms *et al.* 1966; Leng & Felsenfeld, 1966).

It is implicit in the analysis made by Fresco *et al.* (1963) and Felsenfeld & Sandeen (1962) that denaturation leads to a transition from a double-helical to an amorphous form of the polynucleotide chain. This treatment is illustrated in Fig. 1(*a*), where the amorphous form has an extinction, *O*, that does not depend on temperature; the extinction of the double-helical structure, *C*, is also independent of temperature (e.g. $\epsilon_{(P)}$ of DNA is unchanged until denaturation begins) and *OC* is independent of temperature. The observed extinction will be proportional to the fraction, f_{dh}, of the residues that are in double-helical segments:

$$E = af_{dh}\epsilon_{(P)}^{dh} + a(1-f_{dh})\epsilon_{(P)}^{am} \qquad (1)$$

where a is the concentration (moles/l.), $\epsilon_{(P)}$ is the extinction/g.atom of P, and the subscripts and

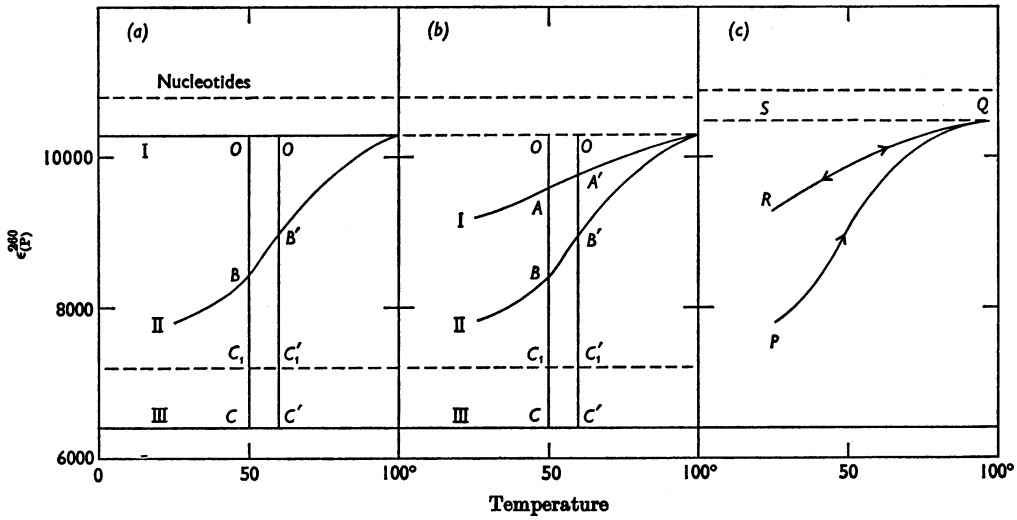

Fig. 1. (*a*) Analysis of the denaturation profile of double-helical secondary structure according to the method of Fresco *et al.* (1963). Curve I shows that the $\epsilon_{(P)}$ of the polynucleotide form is independent of temperature; curve II is the observed dependence of $\epsilon_{(P)}$ on temperature; curve III is the dependence of $\epsilon_{(P)}$ on temperature for the high-molecular-weight double-helical form of RNA having the same average nucleotide composition as the secondary structure giving rise to curve II. The fraction, f_{dh}, of residues in double-helical structures is given by OB/OC, and $\Delta f_{dh}/\Delta T = (BB'/OC)/\Delta T$. (*b*) Analysis of the denaturation profile of double-helical secondary structure taking into account the melting profile of the single-stranded form of RNA (curve I); curves II and III and *C* and C_1 have the same significance as in (*a*): now $f_{dh} = AB/AC$, and $\Delta f_{dh}/\Delta T = [(A'B'/A'C') - (AB/AC)]/\Delta T$. (*c*) Denaturation of unfractionated reticulocyte ribosomal RNA in 1% formaldehyde–0·1 M-potassium phosphate buffer, pH 7·0; curve *PQ* was followed when the solution was first heated to 95°, but curve *QR* was followed on subsequent heating cycles.

superscripts dh and am refer respectively to double-helical and amphorous structures. Hence:

$$f_{dh} = \frac{(a\epsilon^{am}_{(P)} - E)}{(a\epsilon^{am}_{(P)} - a\epsilon^{dh}_{(P)})} = \frac{OB}{OC} \quad (2)$$

and:

$$\Delta f_{dh} = \frac{\Delta E}{a(\epsilon^{am}_{(P)} - \epsilon^{dh}_{(P)})} = \frac{BB'}{OC} \quad (3)$$

The plot of $\epsilon^{am}_{dh} - \epsilon^{dh}_{(P)}$ against wavelength is the denaturation spectrum (per g.atom of P) that is assumed by Fresco *et al.* (1963) to be the same as that found for the double-helical form of high-molecular-weight RNA having the same nucleotide composition as the species being studied.

It is more likely that the polynucleotide forms a single-stranded 'stacked' structure on denaturation. Curve I in Fig. 1(*b*) illustrates the dependence of E of a single-stranded 'stacked' form of a typical RNA molecule on temperature; curve II in Fig. 1(*b*) refers to the observed extinction and curve III in Fig. 1(*b*) to the extinction of the homologous high-molecular-weight double-helical form. If the same nomenclature as before is used, and the superscript s refers to the single-stranded 'stacked' structure, and T_1 and T_2 are different temperatures ($T_2 > T_1$), then:

$$E = (a - af_{dh})\,\epsilon^{s}_{(P)} + af_{dh}\epsilon^{dh}_{(P)} \quad (4)$$

Hence:

$$f_{dh} = \frac{(a\epsilon^{s}_{(P)} - E)}{(a\epsilon^{s}_{(P)} - a\epsilon^{dh}_{(P)})} = \frac{AB}{AC} \quad (5)$$

$$\Delta f_{dh} = \frac{A'B'}{A'C'} - \frac{AB}{AC} \neq \frac{OB}{OC}$$

$$= \frac{(\epsilon^{s(T_1)}_{(P)} - \epsilon^{dh}_{(P)})\,(a\epsilon^{s(T_2)}_{(P)} - E_{T_2}) - (\epsilon^{T_2}_{(P)} - \epsilon^{dh}_{(P)})\,(a^{T_1}_{(P)} - E_{T_1})\epsilon}{a(\epsilon^{s\,T_1)}_{(P)} - \epsilon^{dh}_{(P)})\,(\epsilon^{T_2}_{(P)} - \epsilon^{dh}_{(P)})} \quad (6)$$

If the double-helical segment reverts to a single-stranded 'stacked' structure (curve I in Fig. 1*b*) on denaturation but this effect is neglected, then f_{dh} will be overestimated but $\Delta f_{dh}/\Delta T$ will be underestimated (Fig. 2*a*), and an analysis of the difference in the spectrum found on increasing the temperature from T_1 to T_2 ($\Delta E/\Delta T$) in terms of the nucleotide composition of the double-helical segments denaturing over the range T_1–T_2 will not be valid because eqn. (6) and not eqn. (3) will apply. The nucleotide composition of the double-helical segments may be deduced by means of eqn. (6) if curves I and III in Fig. 1(*b*) are known for each wavelength.

Curve I in Fig. 1(*b*) may be observed after RNA is hydrolysed to fragments about 20 residues long on average (Cox & Kanagalingam, 1967). This limited hydrolysis is sufficient to destroy most of the double-helical secondary structure without appreciably affecting 'stacking', which is hardly chain-length-dependent (Brahms *et al.* 1966).

A more direct measure of curve I in Fig. 1(*b*) was obtained by heating RNA (0·05g./l.) in 1% formaldehyde–0·1M-potassium phosphate buffer, pH 7·0, to 95°. This treatment has been shown to be sufficient to prevent the separated strands of 'phage DNA' from recombining (e.g. Stollar & Grossman, 1962; Thomas & Berns, 1962; but for review see Kit,

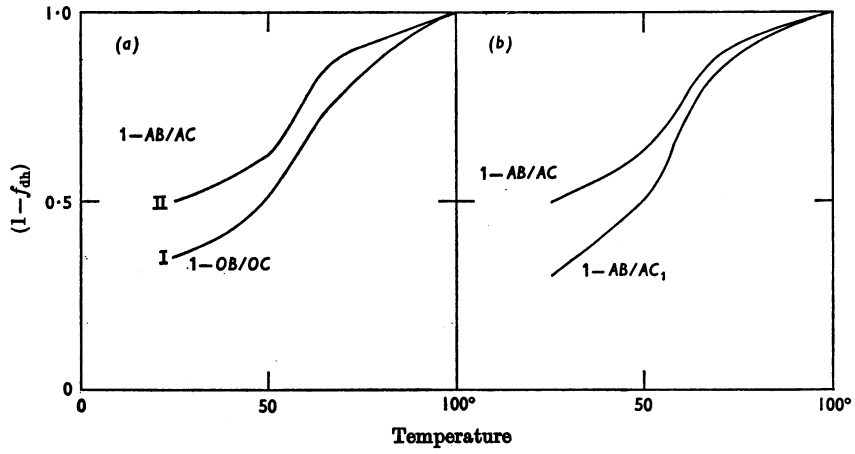

Fig. 2. (*a*) Values of $(1-f_{dh})$ as a function of temperature. Curve I, $(1-f_{dh})$ calculated from curve II in Fig. 1 as in Fig. 1(*a*) ($f_{dh} = OB/OC$); curve II, $(1-f_{dh})$ calculated from curve II in Fig. 1 as in Fig. 1(*b*) ($f_{dh} = AB/AC$). (*b*) Possible errors in $(1-f_{dh})$ due to imprecision in curve III in Fig. 1; f_{dh} was calculated from AB/AC and from AB/AC_1 as indicated in the Figure.

1963). When, for example, unfractionated reticulocyte ribosomal RNA was heated to 95° in 1% formaldehyde–0·1 M-potassium phosphate buffer, pH 7·0, E_{260} increased by 35% (curve PQ in Fig. 1*c*) due partly to the reaction of formaldehyde with the base residues but principally to the loss of secondary structure. When the solution was cooled to 25°, E_{260} decreased by about 14% (curve QR in Fig. 1*c*). The dependence of E_{260} on temperature was then reversible, since curve RQ in Fig. 1(*c*) was followed on subsequent heating cycles. In contrast, when the equivalent mixture of nucleotides, obtained by hydrolysis of RNA with alkali, was heated to 95° in 1% formaldehyde–0·1 M-potassium phosphate buffer, pH 7·0, E_{260} increased by about 6%. Cooling the solution led to a further increase in E_{260} of about 3%, which was reversed when the solution was subsequently brought to 95°. Neither reaction with formaldehyde (Fasman *et al.* 1964) nor hydroxyethylation (Van Holde *et al.* 1965) prevent 'stacking'. Hence we equate curve QR in Fig. 1(*c*) with curve I in Fig. 1(*b*). It may be calculated from the data of Stanley & Bock (1965) that a mixture of di- and tri-nucleotides equivalent to the nucleotide composition of reticulocyte ribosomal RNA will be hypochromic to an extent approaching curve QR in Fig. 1(*c*).

Although curve I in Fig. 1(*b*) may be determined, quantitative analysis of the difference spectrum is still not possible unless $\epsilon_{(P)}^{dh}$ (curve III in Fig. 1*b*) is known. It seems likely that $\epsilon_{(P)}^{dh}$ is dependent on the number of base pairs, N, per double-helical segment (e.g. Rich & Tinoco, 1960; Applequist & Damle, 1965), particularly when N is small ($N < 20$). The imprecision in $\epsilon_{(P)}^{dh}$ is indicated in Figs. 1(*a*) and 1(*b*) as CC_1. The consequent imprecision in $(1-f_{dh})$ calculated from $(1-AB/AC)$ is given in Fig. 2(*b*). If $\epsilon_{(P)}^{dh}$ is not known precisely it is not possible to determine with any certainty the form of the denaturation spectrum because eqn. (6) applies. However, if 'stacking' can be eliminated so that the transition from a partly double-helical form to an amorphous form is observed (Fig. 1*a*), then the form of the denaturation spectrum (i.e. BB' in Fig. 1*a*) can be evaluated even though the absolute change in helical content may not be accurate. Suitable conditions for studying the transition from a double-helical to an amorphous form of the polynucleotide chain were therefore sought.

The $\epsilon_{(P)}$ of polyA is increased when the solution is made 8 M with respect to urea (Fresco & Klemperer, 1959), which also decreases the thermal stability of double-helical structure by about 20°. However, the effect of urea can be countered by increasing the ionic strength tenfold in the case of a double-helical conformation but not if the conformation is a single-stranded 'stacked' structure. Thus 8 M-urea–M-potassium chloride and 4 M-guanidinium chloride

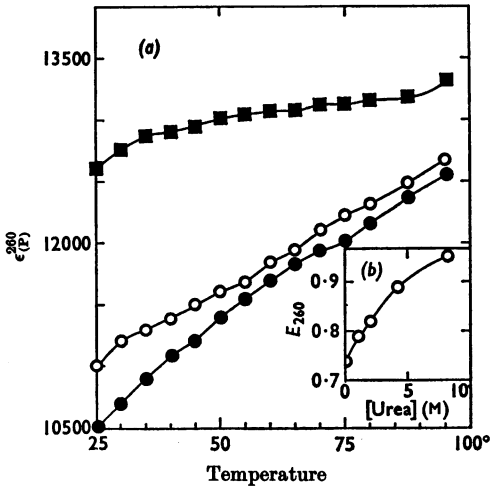

Fig. 3. (*a*) Dependence of $\epsilon_{(P)}$ of oligoA on ionic strength and temperature. ○, 0·01 M-Potassium phosphate buffer, pH 7·0; ●, M-NaCl–0·01 M-potassium phosphate buffer, pH 7·0; ■, 4 M-guanidinium chloride–0·01 M-potassium phosphate buffer, pH 7·0, or 8 M-urea–M-KCl–0·01 M-potassium phosphate buffer, pH 7·0. (*b*) Dependence of E_{260} of oligoA in 0·1 M-potassium phosphate buffer, pH 7·0, on urea concentration.

are solvents in which the transition from a double-helical to an amorphous structure might be observed.

The $\epsilon_{(P)}$ of oligoA (about 6–8 residues long) is hardly dependent on ionic strength (Fig. 3*a*), since increasing the electrolyte concentration from 0·01 M-potassium phosphate buffer, pH 7·0, to M-sodium chloride had little effect on $\epsilon_{(P)}$ above about 50° (cf. Barszcz & Shugar, 1964).

OligoA was dissolved in 0·1 M-potassium phosphate buffer, pH 7·0, containing different amounts of urea. When the concentration of urea was increased to 8·3 M, E_{260} increased from 0·74 to 0·95 at 25° (Fig. 3*b*). A similar increase in E_{260} was noticed (Fig. 3*a*) when the solvent was 8 M-urea–M-potassium chloride–0·01 M-potassium phosphate buffer, pH 7·0, or 4 M-guanidinium chloride–0·01 M-potassium phosphate buffer, pH 7·0. A small increment in E_{260} was found when the temperature was raised to 95°.

Both oligoG and oligoC (6–8 residues long) exhibit hypochromism (Michelson, 1959; see also Pochon & Michelson, 1965; Fasman *et al.* 1964) that is hardly affected by ionic strength (Cox & Kanagalingam, 1967). 'Stacking' is diminished in 4 M-guanidinium chloride–0·01 M-potassium phosphate buffer, pH 7·0, since the values E_{260} 0·955 and E_{280} 0·600 were observed compared with the values E_{260} 0·850 and E_{280} 0·470 found in 0·01–0·1 M-potassium phosphate buffer, pH 7·0. Both E_{260} and

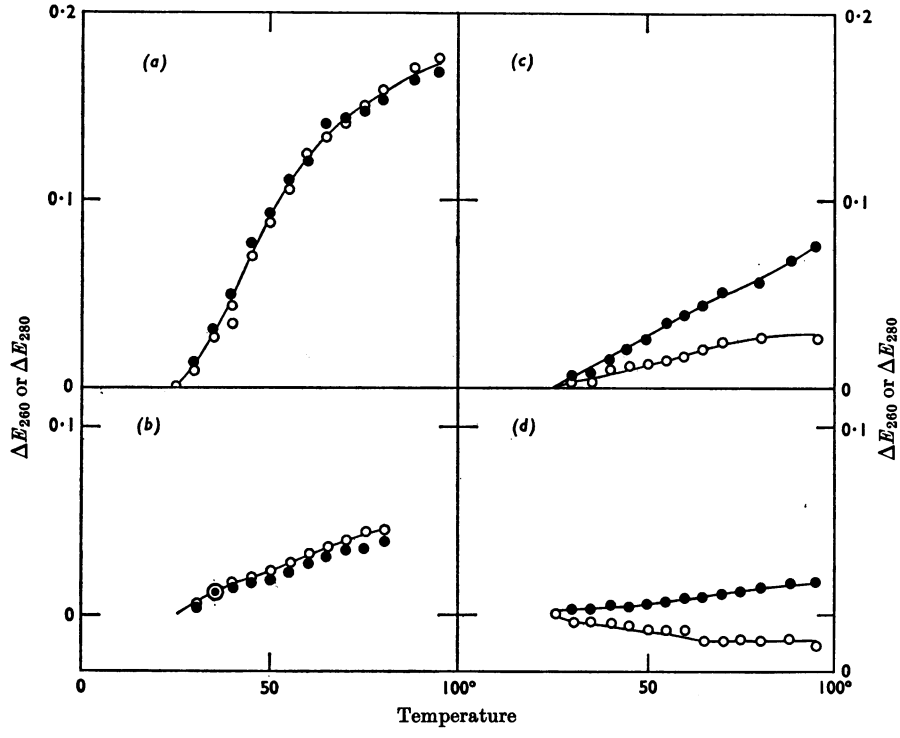

Fig. 4. Effect of guanidinium chloride on the spectra of oligoG and oligoC. (*a*) OligoG in 0·01–0·1 M-potassium phosphate buffer, pH 7·0; (*b*) oligoG in 4 M-guanidinium chloride–0·01 M-potassium phosphate buffer, pH 7·0; (*c*) oligoC in 0·01–0·1 M-potassium phosphate buffer, pH 7·1; (*d*) oligoC in 4 M-guanidinium chloride–0·01 M-potassium phosphate buffer, pH 7·1. ○, ΔE_{260} (E_{260} 1·0 at 25° in each case); ●, ΔE_{280}.

E_{280} increased by about 0·04 unit (E_{260} 1·0 at 25°) when oligoG in 4 M-guanidinium chloride–0·01 M-potassium phosphate buffer, pH 7·0, was heated to 80° (Fig. 4*b*), whereas the increments were 0·18 unit (E_{260} 1·0 at 25°) at both 260 and 280 mμ when 0·1 M-potassium phosphate buffer, pH 7·0, was the solvent (Fig. 4*a*). Similarly, it was found for oligoC (Figs. 4*c* and 4*d*) that E_{260} and E_{280} were higher at 25° in 4 M-guanidinium chloride–0·01 M-potassium phosphate buffer, pH 7·1, than in phosphate buffer alone and that the dependence of E on temperature was considerably diminished when the first-named solvent was used (Figs. 4*c* and 4*d*).

Poly(A + U) was formed by mixing equal volumes of polyA (E_{260} 1·018) and polyU (E_{260} 1·058) in 0·01 M-potassium phosphate buffer, pH 7·0. The extinction of polyU is the same over the range 25–95° (Richards, Flessel & Fresco, 1963) and the melting of polyA is well known; so that the dependence of E_{260} on temperature was calculated, on the basis of the assumption that no complexes are formed, for this mixture of polyA and polyU (curve I in Fig. 5).

When poly(A + U) was heated in 0·01 M-potassium phosphate buffer, pH 7·0, E_{260} increased abruptly by 0·22 over the range 35–40° and increased by a further 0·12 on heating from 45° to 95° according to curve I in Fig. 5. The difference spectrum was essentially the same over the whole range. Increasing the buffer concentration to 0·1 M led to a shift of about 16° in the transition temperature. When the buffer concentration was increased to 0·5 M, the transition range over which the complex dissociated to polyA plus polyU was found to be 65–75°. In contrast with the behaviour noticed at lower ionic strengths E_{280} decreased before the principal transition range and then increased between 65° and 75° to a value slightly above that noticed at 25°. It appears, from the work of Stevens & Felsenfeld (1964), that the decrease in E_{280} signifies that poly(A + U) rearranges to poly(A + 2U) plus polyA, and that poly(A + 2U) subsequently denatures over the range 65–75°. It is evident that heating leads to a transition from a multi-stranded helical form to a single-stranded 'stacked' structure that 'melts' according to curve I in Fig. 5.

When poly(A + U) was added to a solution of 8 M-urea–M-potassium chloride or 4 M-guanidinium

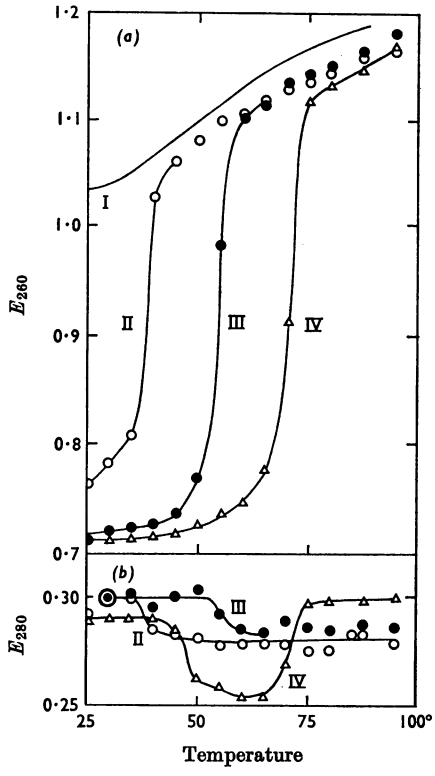

Fig. 5. Effect of ionic strength and temperature on the spectrum of poly(A+U). Equal volumes of polyA (E_{260} 1·018) and polyU (E_{260} 1·058) were mixed to form poly (A+U). Curve I, calculated for a 1:1 mixture of polyA plus polyU assuming no complex-formation; curves II (○), poly(A+U) in 0·01 M-potassium phosphate buffer, pH 7·0; curves III (●), poly(A+U) in 0·1 M-potassium phosphate buffer, pH 7·0; curves IV (△), poly(A+U) in 0·5 M-potassium phosphate buffer, pH 7·0. (*a*) E_{260}; (*b*) E_{280}.

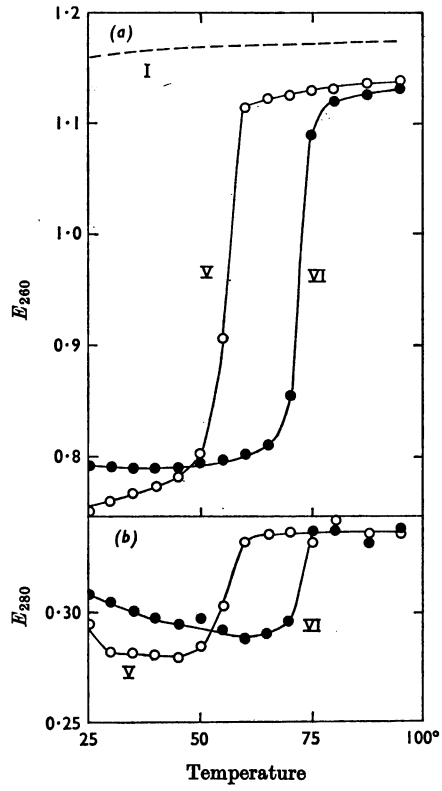

Fig. 6. Effect of guanidinium chloride and urea on the spectrum of poly(A+2U). PolyA (E_{260} 1·018) and polyU (E_{260} 1·058) were mixed in the volume ratio 1:1 to form poly-(A+2U) plus polyA. Curve I, calculated for a 1:1 mixture of polyA plus polyU assuming no complex-formation; curves V (○), poly(A+2U) plus polyA in 8 M-urea–M-KCl–0·01 M-potassium phosphate buffer, pH 7·0; curves VI (●), poly(A+2U) plus polyA in 4 M-guanidinium chloride–0·01 M-potassium phosphate buffer, pH 7·0. (*a*) E_{260}; (*b*) E_{280}.

chloride in 0·01 M-potassium phosphate buffer, pH 7·0, the transition was more clearly defined (Fig. 6). However, the dependence of E_{280} on temperature shows that the system was probably in the form poly(A+2U) plus polyA at 25°. This change in the composition of the complex is attributed to the effect of ionic strength (see Stevens & Felsenfeld, 1964). This change in the nature of the complex is sufficient to account for the value E_{260} 0·79 at 25°, compared with 0·72 at 25° found in 0·1 M-potassium phosphate buffer, pH 7·0. Equimolar mixtures of polyA and polyU in the more usual solvents, e.g. 0·1 M-sodium chloride–mM-magnesium chloride, have identical extinctions whether or not the complex is poly(A+U) or is poly(A+2U) plus polyA (Stevens & Felsenfeld, 1964). The extinction of the mixture poly(A+2U) plus polyA will be higher in 8 M-urea–M-potassium chloride or 4 M-guanidinium chloride–0·01 M-potassium phosphate buffer, pH 7·0, because the extinction of the free polyA is increased (see Fig. 3), owing to a decrease in 'stacking': since half the polyA is free we would expect an increment of 0·08 unit, as observed.

The double-helical form of DNA isolated from rat liver was not affected when the solution was made 4 M with respect to guanidinium chloride, and the mid-point of the thermal transition was found to be 85°, which is close to the value found in standard saline citrate solution (Marmur, Schildkraut & Doty, 1962).

The changes in E_{260} and E_{280} found on heating unfractionated t-RNA in 0·1 M-potassium phosphate buffer, pH 7·0, are given in Fig. 7(*a*): the extinction increased over the range 25–95° from

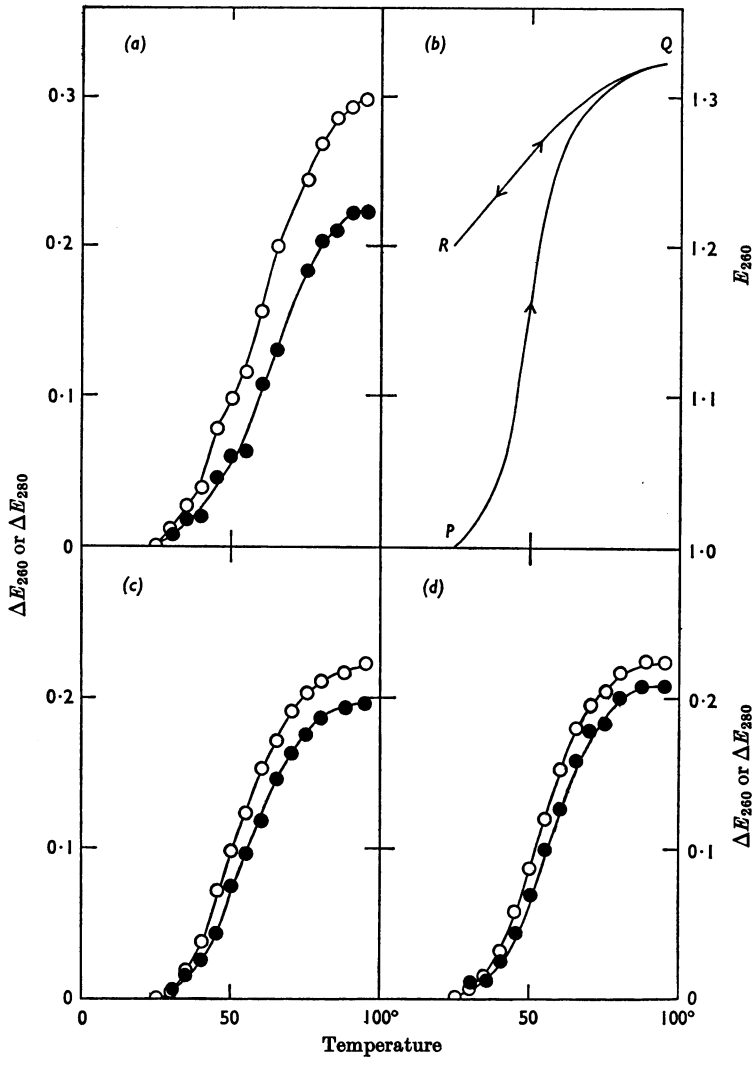

Fig. 7. Denaturation of unfractionated t-RNA from *E. coli* in different solvents. (*a*) Unfractionated *E. coli* t-RNA in 0·1 M-potassium phosphate buffer, pH 7·0. (*b*) Unfractionated *E. coli* t-RNA in 1% formaldehyde–0·1 M-potassium phosphate buffer, pH 7·0; curve *PQ* was followed when the solution was first heated to 95°, but curve *QR* was followed on subsequent heating cycles. (*c*) Unfractionated *E. coli* t-RNA in 8 M-urea–M-KCl–0·01 M-potassium phosphate buffer, pH 7·0. (*d*) Unfractionated *E. coli* t-RNA in 4 M-guanidinium chloride–0·01 M-potassium phosphate buffer, pH 7·0. In (*a*), (*c*) and (*d*): ○, ΔE_{260} (E_{260} 1·0 at 25° in each case); ●, ΔE_{280}.

1·00 to 1·30 at 260mμ and from 0·45 to 0·67 at 280mμ. When the solvent was 8 M-urea–M-potassium chloride–0·01 M-potassium phosphate buffer, pH 7·0, E_{260} and E_{280} respectively were about 8% and 16% greater at 25° than in 0·1 M-potassium phosphate buffer, pH 7·0; and when the solution was heated the total increments in E were decreased to about 22% for E_{260} and about 40% for E_{280} (Fig. 7*c*), although the temperature at the mid-point of the transition was close (55°) to that found (59°) in 0·1 M-potassium phosphate buffer, pH 7·0. Similar changes in the spectrum were found (Fig. 7*d*) in 4 M-guanidinium chloride–0·01 M-potassium phosphate buffer, pH 7·0, as in 8 M-urea–M-potassium chloride–0·01 M-potassium phosphate buffer, pH 7·0 (Fig. 7*c*). It appears that single-stranded 'stacked' residues make a substantial contribution to the secondary structure of *E. coli* t-RNA in 0·1 M-potassium phosphate buffer, pH 7·0, at 25° [cf. poly(A + 2U) plus polyA], as suggested

previously (Fasman *et al.* 1965; Cantor *et al.* 1966; Michelson, Ulbricht, Emerson & Swan, 1966; Kay & Oikawa, 1966). [The extent of 'stacking' after RNA was treated with formaldehyde is apparent from Fig. 7(*b*).] Adenine and guanine residues may contribute to 'stacking' since both E_{260} and E_{280} are affected by the addition of urea. It is inferred that the fraction of residues in double-helical segments is considerably less than earlier values derived from the denaturation spectrum (Felsenfeld & Cantoni, 1964; Fresco *et al.* 1963). The ratio $\Delta E_{280}/\Delta E_{260}$ measures the fraction of guanine and cytosine residues in double-helical segments (Felsenfeld & Sandeen, 1962; Fresco *et al.* 1963; Cox, 1966*a*,*b*). When 4M-guanidinium chloride–0·01M-potassium phosphate buffer, pH 7·0, or 8M-urea–M-potassium chloride–0·01M-potassium phosphate buffer, pH 7·0, was the solvent $\Delta E_{280}/\Delta E_{260}$ was about 0·90, showing that the double-helical segments are rich in guanine and cytosine residues. The lower value of $\Delta E_{280}/\Delta E_{260}$, namely 0·74, found in 0·1M-potassium phosphate buffer, pH 7·0, is attributed to the presence of 'stacked' adenine residues that 'melt' over the same temperature range as the double-helical segments. Although 'stacking' might contribute to the stability of the double-helical segments this effect is apparently not large since T_m (i.e. the temperature at which 50% of the increase in E is obtained on heating oligo- or polynucleotides) is not markedly affected by introducing urea (cf. Figs. 7*a* and 7*b*). The double-helical segments must be short (about 5 base pairs) or grossly imperfect, because high-molecular-weight RNA in the double-helical form 'melts' at about 100° (e.g. Gomatos & Tamm, 1963). Studies of model systems (Doty, 1961; Lipsett, Heppel & Bradley, 1961; Lipsett, 1964) have shown that T_m is inversely proportional to the number of base pairs, N, of the double-helical segment. It was suggested (Cox, 1966*a*) that this relation may be generally applicable to RNA. It is only when N is small that T_m is appreciably less than that of the high-molecular-weight molecule.

Thus the changes in the spectrum of t-RNA found on heating in 8M-urea–M-potassium chloride or 4M-guanidinium chloride in 0·01M-potassium phosphate buffer, pH 7·0, fit more closely the models for the secondary structure based on a knowledge of the nucleotide sequence [i.e. approx. 50% of residues form base pairs, which principally involve guanine and cytosine residues, and the helical segments are about 5 base pairs in length (Holley *et al.* 1965; Vournakis & Scheraga, 1966; Cantor *et al.* 1966)] than the conclusions of Felsenfeld & Cantoni (1964), Fresco *et al.* (1963) and Englander & Englander (1965).

It has been shown that in solutions of high ionic strength or in the presence of Mg^{2+} the 'melting' profile of t-RNA is sharpened (for review see Brown, 1963). This is to be expected if the partly

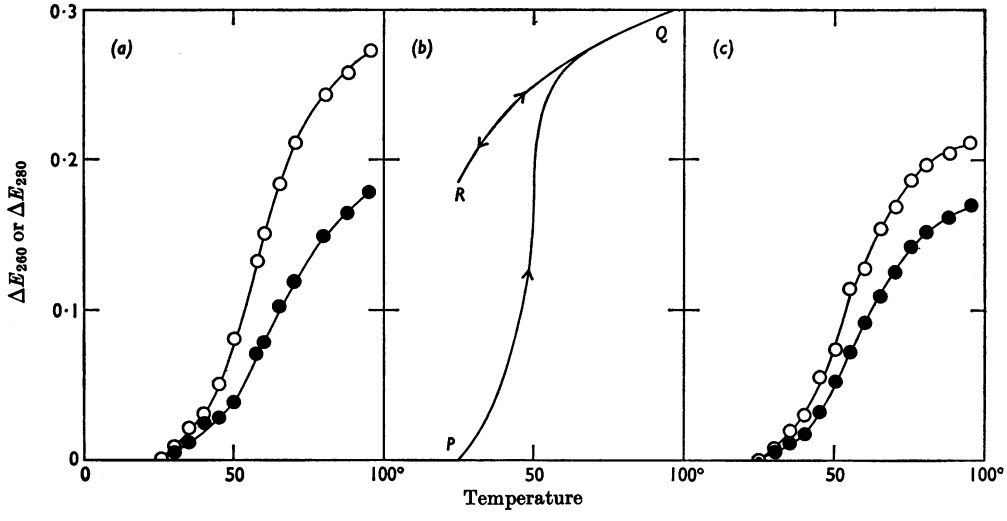

Fig. 8. Denaturation of RNA from the smaller ribosomal sub-unit isolated from rabbit reticulocytes. (*a*) RNA from the smaller ribosomal sub-unit from rabbit reticulocytes in 0·1M-potassium phosphate buffer, pH 7·0. (*b*) RNA from the smaller ribosomal sub-unit from rabbit reticulocytes in 1% formaldehyde–0·1M-potassium phosphate buffer, pH 7·0; curve *PQ* was observed on first heating to 95°, but curve *QR* was followed on subsequent heating cycles. (*c*) RNA from the smaller ribosomal sub-unit from rabbit reticulocytes in 4M-guanidinium chloride–0·01M-potassium phosphate buffer, pH 7·0. In (*a*) and (*c*): ○, ΔE_{260}; ●, ΔE_{280}; in (*b*), the curves are for ΔE_{260}. In each case $E_{260}=1·0$ at 25°.

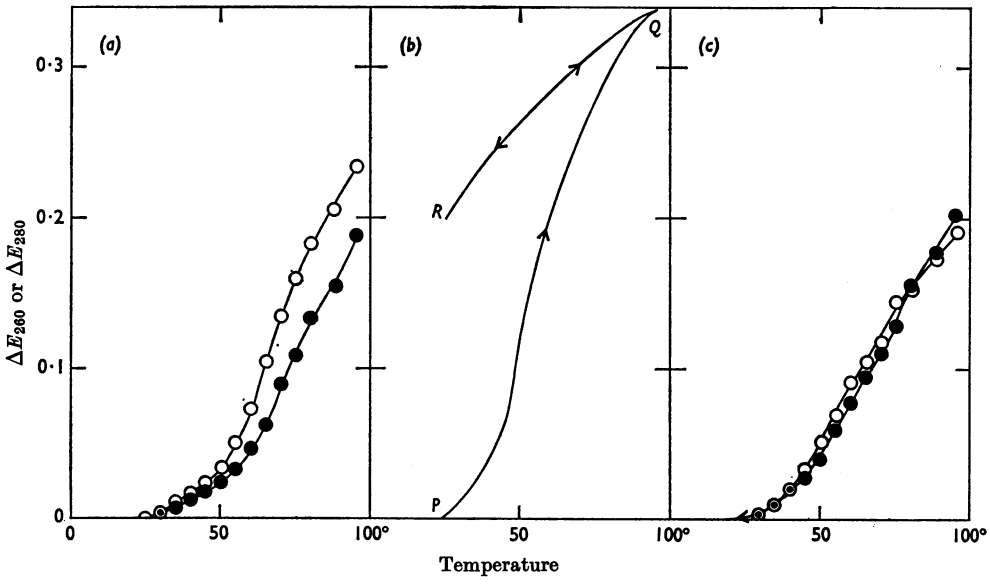

Fig. 9. Denaturation of RNA from the larger ribosomal sub-unit isolated from rabbit recitulocytes. (*a*) RNA from the larger ribosomal sub-unit from rabbit reticulocytes in 0·1 M-potassium phosphate buffer, pH 7·0. (*b*) RNA from the larger ribosomal sub-unit from rabbit reticulocytes in 1% formaldehyde–0·1 M-potassium phosphate buffer, pH 7·0; curve *PQ* was observed when the solution was first heated to 95°, but curve *QR* was followed on subsequent heating cycles. (*c*) RNA from the larger ribosomal sub-unit from rabbit reticulocytes in 4 M-guanidinium chloride–0·01 M-potassium phosphate buffer, pH 7·0. In (*a*) and (*c*): ○, ΔE_{260}; ●, ΔE_{280}; in (*b*), the curves are for ΔE_{260}. In each case $E_{260} = 1{\cdot}0$ at 25°.

double-helical structure reverts to a single-stranded 'stacked' structure on denaturation. Since 'stacking' is likely to be decreased at higher temperatures, increasing T_m by increasing the ionic strength will lead to a closer approach to the transition to an amorphous form of the polynucleotide.

The spectrum of RNA from the smaller ribosomal sub-unit isolated from rabbit reticulocytes was examined in different solvents (Figs. 8*a*, 8*b* and 8*c*). A broad transition was noted in 0·1 M-potassium phosphate buffer, pH 7·0, and $\Delta E_{280}/\Delta E_{260}$ was found to be 0·65. In 1% formaldehyde–0·1 M-potassium phosphate buffer, pH 7·0, curve *PQ* in Fig. 8(*b*) was followed on heating to 95°. The curve *QR* in Fig. 8(*b*), which was followed on subsequent heating cycles, corresponds to curve I in Fig. 1(*b*). The total increment in E_{260} decreased from 27% (Fig. 8*a*) to 21% and $\Delta E_{280}/\Delta E_{260}$ increased from 0·655 to 0·81 when the solvent was 4 M-guanidinium chloride–0·01 M-potassium phosphate buffer, pH 7·0 (Fig. 8*c*). The transition range was more clearly defined in the latter solvent and 'melting' was complete by 80°. It is concluded that 'stacking' is appreciable at 25° in 0·1 M-potassium phosphate buffer, pH 7·0, and that *N* is small (cf. Cox, 1966*a*; Cox & Kanagalingam, 1967).

RNA from the larger ribosomal sub-unit isolated from rabbit reticulocytes was less affected by guanidinium chloride, although $\Delta E_{280}/\Delta E_{260}$ increased from 0·81 in 0·1 M-potassium phosphate buffer, pH 7·0, to 1·05 in 4 M-guanidinium chloride–0·01 M-potassium phosphate buffer, pH 7·0. The increment in E_{260} was decreased from 0·235 (Fig. 9*a*) to 0·195 (Fig. 9*c*). The increments in both E_{260} and E_{280} noted above 75° in 0·1 M-potassium phosphate buffer, pH 7·0, persisted in guanidinium chloride, showing that relatively stable double-helical structure rich in guanine and cytosine residues is present in this species of RNA. A similar conclusion was reached on the basis of the effect of pH on the denaturation spectrum (Cox, 1966*a*) of unfractionated reticulocyte ribosomal RNA, which principally reflects the properties of the 30s component. The tendency in 0·1 M-potassium phosphate buffer, pH 7·0, of the residues to 'stack' was revealed (curve *QR* in Fig. 9*b*) after RNA had reacted with formaldehyde (curve *PQ* in Fig. 9*b*).

In conclusion, we believe that 'stacking' may contribute to the secondary structure of both t-RNA and ribosomal RNA at 25°, and may affect the denaturation spectrum of RNA unless precautions are taken to observe the transition from the partly double-helical to the amorphous form of the polynucleotide.

EXPERIMENTAL

Materials. OligoA, oligoC and oligoG (6–10 residues long) were given by Dr A. Michelson. Rat-liver DNA was given by Dr C. Gonzales. PolyA and polyU were purchased from Miles Chemical Corp., Elkhart, Ind., U.S.A., and were used without further purification.

Unfractionated *E. coli* t-RNA (given by Dr E. M. Martin) was isolated by the method of Martin, Yegian & Stent (1963) and purified by chromatography on DEAE-cellulose as described by Cannon (1966).

Reticulocyte ribosomal RNA (given by Dr Hannah Gould) was isolated by the method of Cox (1966c) from ribosomal sub-units fractionated by zone centrifugation (Klucis & Gould, 1966). Guanidinium chloride was prepared from A.R. guanidinium carbonate by neutralization with conc. HCl. The solution was treated with Norit and freed from both CO_2 and u.v.-absorbing impurities by heating to 90°.

Urea was recrystallized from aqueous ethanol.

A.R. formaldehyde was used without further purification.

Measurement of $\epsilon_{(P)}$. OligoA was hydrolysed with alkali, pH13, and the concentration of adenine residues was calculated by relating the observed extinction to a value of $15400/\epsilon_{(P)}$ of AMP.

Spectrophotometry. A Unicam SP.700 spectrophotometer was used for all measurements. The sample-cell holder was replaced by an electrically heated copper block (described by Cox, 1963*a*).

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