A Spectrophotometric Study of the Secondary Structure of Ribonucleic Acid Isolated from the Smaller and Larger Ribosomal Subparticles of Rabbit Reticulocytes

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The spectrum of RNA from the smaller and larger subparticles of rabbit reticulocyte ribosomes was studied as a function of pH, ionic strength, urea concentration and temperature. It was inferred that both RNA species form short double-helical segments of not more than about 10 base-pairs in length. Not more than about 70% of the base residues may be located in double-helical segments. RNA from the larger subparticle is richer in guanine and cytosine residues and its secondary structure is the more stable. These conclusions are based on the use of double-helical RNA from virus-like particles and of unfractionated *Escherichia coli* tRNA as model systems.

Within the ribosome the RNA species appear to have a partly double-helical secondary structure that is retained in solution after the protein moiety is removed (for reviews see Arnstein, 1963; Petermann, 1964; Cox, 1969). Thus, a knowledge of the secondary structure of rRNA in solution is relevant to understanding the conformation of rRNA *in vivo*. The secondary structure of RNA from reticulocyte ribosomes was treated as an assembly of short hairpin loops (about 20-40 nucleotides long) formed by the polynucleotide chain folding back upon itself (Cox & Kanagalingam, 1967a; Cox, Gould & Kanagalingam, 1968). When unfractionated rabbit reticulocyte RNA was examined some properties of the double-helical segments of the hairpin loops were deduced from a study of the changes in the u.v.-absorption spectrum as a function of pH, electrolyte concentration and temperature (Cox, 1966a,b). The number of base-pairs/double-helical segment was estimated to be between four and 17, and the double-helical segments appeared to differ appreciably in nucleotide composition as well as in chain length. The properties of unfractionated reticulocyte rRNA reflect mainly the properties of the so-called 30S RNA species that is a component of the larger ribosomal subparticle. It was subsequently shown that the tendency of base residues to stack one upon another in single-stranded regions should be taken into account in interpreting the changes in the spectrum of both 16S and 30S rRNA (Cox & Kanagalingam, 1967b, 1968). The aim of this work (which was briefly described earlier; Cox, 1967) is

to compare and contrast the secondary structures of the 16S and 30S rRNA species isolated from the fractionated ribosomal subparticles as revealed by spectrophotometric measurements.

THEORETICAL

The basis of the analysis was described by Felsenfeld & Sandeen (1962) and Fresco, Klotz & Richards (1963), who showed that the nucleotide composition of native DNA could be calculated from the difference in the u.v.-absorption spectrum that is found on denaturation in moderately concentrated neutral electrolyte solutions (e.g. standard saline-citrate buffer).

In principle the same methods can be applied to single-stranded polynucleotides such as rRNA or tRNA, which have a partly double-helical character (see also Cox, 1966a,b). However, one difficulty that arises is that, although a change in extinction is generally 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 is always due to denaturation of double-helical secondary structure. It is known that base-residues have a tendency to stack one upon another even in single-stranded regions of a polynucleotide. The factor that complicates the analysis of the spectrum is that spectral changes found when the residues become unstacked are similar to those found on the breakdown of double-helical structures.

The principal possible conformations of a single-stranded polynucleotide are illustrated in Scheme 1 (for review see Cox, 1968a). A polynucleotide may be amorphous (Scheme 1), i.e. the base residues have no preferred orientation with respect to one another. It is inferred that increasing the temperature will not affect

Scheme 1. Equilibria between the partly double-helical, single-stranded 'stacked' and amorphous forms of a polynucleotide. Suffices dh, s and am indicate double-helical, single-stranded stacked and amorphous forms respectively. The equilibrium constant K' pertains to the titration of the $\text{NH}_{(1)}\text{-CO}_{(6)}$ group of uracil and guanine residues with alkali at constant temperature; K'' pertains to the titration of $\text{N}_{(1)}=\text{C}_{(6)}\text{-NH}_2$ group of adenine and cytosine and the $\text{C}_{(6)}\text{-NH}_2$ group of guanine with acid at constant temperature. + and - indicate the ionized forms of the polynucleotide in acidic and alkaline solutions respectively. The equilibrium between the partly double-helical and single-stranded stacked forms at constant pH is defined by k_1 , k_2 or k_3 according to the state of ionization of the base residues. The corresponding constants for the equilibrium between the single-stranded stacked and amorphous forms are k'_1 , k'_2 and k'_3 .

the spectrum because the conformation is unlikely to be altered.

Polynucleotides sometimes approach the amorphous state at high temperatures. However, the base-residues have an increasing tendency to interact with one another as the temperature is lowered. The term 'stacking' has been used to describe this interaction, because the flat purine or pyrimidine rings tend to 'stack' like a pile of pennies with the plane of the base-residues perpendicular to the helix axis (Brahms, Michelson & Van Holde, 1966). The distance between base-residues appears to be about 3 Å with a translation of 30–45° between adjacent residues, there being 8–12 residues/turn of the helix (Bush & Tinoco, 1967). Interaction takes place principally between nearest neighbours, so that the dinucleotide may be regarded as a model for the single-stranded polymer (see, e.g., Van Holde, Brahms & Michelson, 1965).

A simple and relatively precise interpretation of the dependence of $\epsilon_{(P)}$ on temperature is that there is an equilibrium between the amorphous and single-stranded 'stacked' forms that is given by the equation:

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

where f_s is the fraction of residues that are stacked, $(1-f_s)$ is the fraction of residues that are amorphous, ϵ_{am}^0 and ϵ_s^0 respectively are the values of $\epsilon_{(P)}$ when the

polynucleotide is entirely amorphous ($f_s = 0$) or entirely in the 'stacked' conformation ($f_s = 1$), ϵ_s is the observed $\epsilon_{(P)}$ value when the fraction of stacked residues is f_s and K is the equilibrium constant (see Leng & Felsenfeld, 1966; Brahms *et al.* 1966; Cox & Kanagalingam, 1968; Richards, 1968). It is implicit in eqn. (1) that the increase in extinction on increasing the temperature is proportional to the increase in the fraction of residues in the amorphous form.

Many species of RNA are entirely single-stranded at high temperatures and low ionic strengths, but as the temperature is decreased or the ionic strength is increased the polynucleotide may fold upon itself, forming short hairpin loops that are stabilized by interaction principally between complementary base-pairs, located on different segments of the same molecule. Three parameters, namely the number, N , of base-pairs per loop, the number, b , of unpaired residues per loop, and the number, c , of unpaired residues that link one hairpin loop to another, serve to characterize the partly double-helical structure.

The optical properties of the partly double-helical form appear to be the sum of the properties of the double-helical and single-stranded regions; the changes in the spectrum were analysed on the basis of this assumption.

The temperature range over which the increment in $\epsilon_{(P)}$ due to the transition from a multi-helical to a single-stranded form takes place depends on the number, N , of base-pairs per multi-helix and the nucleotide composition of the polymer. The temperature at which 50% of the increment in $\epsilon_{(P)}$ is found is termed T_m (expressed in °K).

Studies with model compounds (e.g. the interaction of oligonucleotides with their complementary polynucleotides) have shown that when N is greater than 20, T_m is scarcely dependent on N . Several attempts to relate T_m and N have been made. Empirical relations (Doty, 1961; Lipsett, Heppel & Bradley, 1961; Lipsett, 1964) were generalized (eqn. 2) and applied to RNA (Cox, 1966a,b); it was implicitly assumed that the unpaired residues within a loop did not affect T_m :

$$T_m = T_{m(\infty)} - 172/N \quad (2)$$

where T_m refers to the species being investigated and $T_{m(\infty)}$ to the reference compound, which has the same chemical features except that $N \rightarrow \infty$. Although the nucleotide sequence of a short double-helical segment may influence T_m (Bautz & Bautz, 1964), this was neglected in view of the dominant effect of chain length.

Relations based on theoretical treatments are given in eqns. (3) and (4). Eqn. (3) was derived (Applequist & Damle, 1965) to account for interactions between oligo A species of defined size. Thus:

$$1/T_m = R\gamma/N\Delta H + 1/T_{m(\infty)} \quad (3)$$

where γ is a concentration parameter and ΔH (which is assumed to be independent of temperature) is the enthalpy/mol of nucleotide pairs for increasing the length of the helix by one base-pair.

Eqn. (4) was developed (Kallenbach, 1968) for short hairpin loops of defined size and takes into account the effect of the b unpaired residues on T_m . Thus:

$$1/T_m = 1/T_{m(\infty)} - (R/N\Delta H) \ln[\tau(b/2)1.5] \quad (4)$$

where τ is the equilibrium constant for 'stacking' two adjacent base-pairs upon each other. Eqn. (4) was rearranged [eqn. (5)] to give N explicitly; and $T_{m(\infty)}$ was related to $T_{m(A\cdot U)}$ and $T_{m(G\cdot C)}$ [the values of $T_{m(\infty)}$ for poly (A-U) and poly G·polyC respectively] and the mole fraction $f_{(G\cdot C)}$ of G·C base-pairs:

$$N = \frac{1.5 \ln b + \ln \tau - 1.5 \ln 2}{(1/T_{m(A\cdot U)} - 1/T_m) \Delta H/R + f_{(G\cdot C)} \ln K} \quad (5)$$

where $\ln K = \Delta H/R(1/T_{m(G\cdot C)} - 1/T_{m(A\cdot U)})$. Since $1/T_{m(A\cdot U)}$ and $\ln K$ are known, T_m is the observed value for a double helix of N base-pairs, $f_{(G\cdot C)}$ may be derived from the difference spectrum (see below) and τ is known approximately (Kallenbach, 1968) then N may be evaluated as a function of b . ΔH is assumed to be independent of temperature.

It is well known (for review see Marmur, Schildkraut & Doty, 1962) that the T_m of DNA depends on the mole fraction of G·C base-pairs. Increasing knowledge of the properties of high-molecular-weight double-stranded

'Helical content' and the nucleotide composition of double-helical segments. The fraction of residues located in double-helical segments [$f_{dh} = \epsilon_{2N}/\epsilon(2N+b+c)$] is given (Boedtker, 1967; Cox & Kanagalingam, 1967b) by the equation:

$$f_{dh} = (\epsilon_s - \epsilon)/(\epsilon_s - \epsilon_{dh}^0) \quad (8)$$

ϵ_s is dependent on f_s , which varies with temperature. When f_s is small $\epsilon_s \rightarrow \epsilon_{am}^0$.

The nucleotide composition of the double-helical segments 'melting' over a particular temperature range can be deduced from the difference in the spectrum at the two temperatures provided that the extent of 'stacking' along the single strand is small (Felsenfeld & Sandeen, 1962; Fresco *et al.* 1963; Cox, 1966a,b). On the basis of the study of the denaturation spectrum of the polynucleotides poly A·poly U and poly(G,C) the relative proportion [$\delta f_{(A\cdot U)}/\delta f_{(G\cdot C)}$] of base-pairs formed between adenine and uracil residues (A·U base-pairs) and between guanine and cytosine residues (G·C base-pairs) is given by the equation (Cox, 1966a,b):

$$\delta f_{(A\cdot U)}/\delta f_{(G\cdot C)} = \Delta\epsilon_{280(G\cdot C)}/\Delta\epsilon_{260(A\cdot U)}(\Delta\epsilon_{260}/\Delta\epsilon_{280}) - \Delta\epsilon_{260(G\cdot C)}/\Delta\epsilon_{260(A\cdot U)} \quad (9)$$

RNA has enabled the relation between $1/T_m$ and $f_{(G\cdot C)}$ to be established (cf. Kallenbach, 1968) for standard saline-citrate (15mM-NaCl-0.15M sodium citrate buffer, pH7) as solvent, namely:

$$1/T_m = 2.93 \times 10^{-3} - 0.51 \times 10^{-3} f_{(G\cdot C)} \quad (6)$$

Since T_m in other solvents can be related to T_m in standard saline-citrate, eqn. (6) can be adapted for a variety of experimental conditions. Thus the T_m in 4M-guanidinium chloride was found to be about 11°C less than in standard saline-citrate and the T_m in 0.1M-sodium phosphate and 0.01M-sodium phosphate buffer respectively was smaller by 9°C and 20°C than that in standard saline-citrate.

Breadth of the 'melting' profile. The temperature range over which a single species of double helix 'melts' depends on N . According to Kallenbach (1968) the slope of the melting profile at $T = T_m$ is linearly related to N by the equation:

$$\left(\frac{\partial f_{dh}}{\partial T}\right)_{T=T_m} = N(\Delta H/4R T_m^2) \quad (7)$$

where f_{dh} is the fraction of residues forming base-pairs. The interquartile range (i.e. the temperature range over which ϵ changes from 0.25 to 0.75 of the total increment owing to the transition from double-helical to single-stranded forms) provides a convenient measure of the

where $\Delta\epsilon_{280(G\cdot C)} = 3070$, $\Delta\epsilon_{260(A\cdot U)} = 3900$, $\Delta\epsilon_{260(G\cdot C)} = 1800$. $\Delta\epsilon_{(A\cdot U)}$ and $\Delta\epsilon_{(G\cdot C)}$ are the increments in $\epsilon_{(P)}$ due to the 'melting' of A·U and G·C base-pairs respectively, i.e. $\epsilon_{am}^0(A\cdot U) - \epsilon_{dh}^0(A\cdot U)$ etc. $f_{(A\cdot U)}$ and $f_{(G\cdot C)}$ are respectively the fractions of the total base-residues forming A·U and G·C base-pairs. $\delta f_{(A\cdot U)}$ and $\delta f_{(G\cdot C)}$ are the changes in $f_{(A\cdot U)}$ and $f_{(G\cdot C)}$ leading to the observed increments $\Delta\epsilon$.

The essence of the method is that there is an increase in ϵ_{260} but not in ϵ_{280} when A·U base-pairs denature whereas both ϵ_{260} and ϵ_{280} increase when G·C base-pairs denature. Thus $\Delta\epsilon_{260}$ provides a measure of the denaturation of both A·U and G·C base-pairs whereas $\Delta\epsilon_{280}$ measures solely the denaturation of G·C base-pairs.

From a study (see Cox, 1967) of the difference in the spectrum of several different species of RNA that were heated from 25°C to 95°C in 0.1M-potassium phosphate buffer, pH7.6, the overall nucleotide composition was related to $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ by the equation:

$$(A+U)/(G+C) = 0.75 (\epsilon_{260}/\epsilon_{280}) - 0.36 \quad (10)$$

On the basis of the assumption that the nucleotide composition of the double-helical segment is directly related to the overall nucleotide composition [i.e. $\delta f_{(A\cdot U)}/\delta f_{(G\cdot C)} = (A+U)/(G+C)$] eqn. (10) is in good agreement with eqn. (9) and provides further evidence for its validity. Eqn. (9) also applies in principle to the analysis of hydrolysis difference spectra. The appropriate equation is eqn. (9a):

$$\delta f_{(A\cdot U)}/\delta f_{(G\cdot C)} = \Delta\epsilon_{260}/\Delta\epsilon_{280} (\Delta\epsilon_{280}^H(G\cdot C)/\Delta\epsilon_{260}^H(A\cdot U)) - \Delta\epsilon_{280}^H(G\cdot C)/\Delta\epsilon_{260}^H(A\cdot U) \quad (9a)$$

breadth of the 'melting' range. The interquartile range calculated (Kallenbach, 1968) for $N = 5$ is about 17°C for both A·U and G·C base-pairs, comparable with the range found for complexes ($A_5 \cdot A_5$) formed between oligoadenylate molecules that are five residues long (data of Applequist & Damle, 1965).

where $\Delta\epsilon_{(A\cdot U)}^H = \epsilon_{(nucleotides)} - \epsilon_{dh}^0(A\cdot U)$; and $\Delta\epsilon_{(G\cdot C)}^H = \epsilon_{(nucleotides)} - \epsilon_{dh}^0(G\cdot C)$. The reported values are $\Delta\epsilon_{260}^H(A\cdot U) = 6000$ (Richards & Simpkins, 1968) and $\Delta\epsilon_{260}^H(G\cdot C) = 2000$ and $\Delta\epsilon_{280}^H(G\cdot C) = 4800$ (calculated from Haselkorn & Fox, 1965), which lead to a slope of 0.73 [compared with 0.79 for eqn. (9) and 0.75 for eqn. (10)] and to an intercept of

−0.33 [compared with −0.46 for eqn. (9) and −0.36 for eqn. (10)]. The agreement between eqns. (9) and (9a) is satisfactory. When the 'melting' properties of two species of double-helical RNA isolated from a virus that grows in mycelia of *Penicillium chrysogenum* (given by Dr Elizabeth Sutherland) were studied values of $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ of 2.8 and 1.9 respectively were found. The values of (G+C) content calculated from eqn. (9) were 36 and 51% compared with values of 37 and 51% obtained by chemical analysis. Thus eqn. (9) is substantially correct, although minor revision might be necessary when results for other species of double-helical RNA become available.

When 4M-guanidinium chloride was the solvent the equation analogous to eqn. (10) was found to be:

$$\delta f_{(A \cdot U)} / \delta f_{(G \cdot C)} = 0.85 (\Delta\epsilon_{260} / \Delta\epsilon_{280}) - 0.39 \quad (11)$$

The difference between eqns. (9) and (11) is attributed to two effects, namely: (i) a small perturbation of the spectrum due to 4M-guanidinium chloride; (ii) a closer approach to the ideal case (i.e. a transition from a partly double-helical conformation to an amorphous single strand) owing to the ability of 4M-guanidinium chloride to diminish stacking in single-stranded regions (Cox & Kanagalingam, 1967b).

The value of ϵ_{dh} can be calculated for any double-helical structure from eqn. (12), which is readily derived from eqn. (9):

$$\epsilon_{dh} = \epsilon_{am} - [\Delta\epsilon_{(G \cdot C)} \cdot f_{(G \cdot C)} + \Delta\epsilon_{(A \cdot U)} \cdot f_{(A \cdot U)}] \quad (12)$$

At 260nm $\Delta\epsilon_{(A \cdot U)}$ and $\Delta\epsilon_{(G \cdot C)}$ were taken to be 3900 units (Fresco *et al.* 1963) and 1800 units (Cox, 1966a,b) respectively (solvent 0.01–0.1M-sodium phosphate buffer, pH7.6) and 3650 and 1700 units respectively (solvent 4M-guanidinium chloride–0.01M-sodium phosphate buffer, pH7.2). Good agreement between the observed and calculated values were found for the species of double-helical viral RNA mentioned above. The values observed in 0.01M-sodium phosphate buffer were 3250 and 2800 units respectively compared with the values of 3150 and 2860 units calculated by means of eqn. (12) for RNA of (G+C) contents 37% and 51%.

In solutions of 4M-guanidinium chloride the observed values of $\epsilon_{am}^0 - \epsilon_{dh}^0$ were respectively 3350 and 3150 compared with the calculated values of 3100 and 2850. Another estimate of ϵ_{dh}^0 can be obtained from eqn. (12a), derived from eqn. (9a):

$$\epsilon_{dh}^0 = \epsilon_{(nucleotides)} - [\Delta\epsilon_{(G \cdot C)}^H \cdot f_{(G \cdot C)} + \Delta\epsilon_{(A \cdot U)}^H \cdot f_{(A \cdot U)}] \quad (12a)$$

Eqn. (12) is based on the assumption that $\Delta\epsilon_{(G \cdot C)}$ and $\Delta\epsilon_{(A \cdot U)}$ are independent of nucleotide sequence (see Richards, 1968). It is possible that $\Delta\epsilon_{(A \cdot U)}$ and $\Delta\epsilon_{(G \cdot C)}$ are different for base-pairs at the ends of a double-helical segment (e.g. Rich & Tinoco, 1960). An allowance of a 10% decrease in $\epsilon_{am} - \epsilon_{dh}$ is made for this effect based on the results of Applegate & Damle (1965). The values of ϵ_{dh}^0 calculated by means of eqn. (12) for the RNA from the smaller and larger subparticles in the entirely double-helical conformation are 6900 and 7000 respectively. Allowing a decrease of 10% in $\Delta\epsilon_{(A \cdot U)}$ and $\Delta\epsilon_{(G \cdot C)}$ for the dependence on chain length then the appropriate value of ϵ_{dh} is estimated to lie within the ranges 6700–7100 and 7000–7250 for RNA from the smaller and larger subparticles respectively. Hence the fraction of residues,

f_{dh} , that are base-paired which 'melt' over the temperature range ΔT leading to an increase $\Delta\epsilon$ in extinction is given by the equation:

$$\delta f_{dh} = \Delta\epsilon / [\epsilon_{am}^0 - \Delta\epsilon_{(G \cdot C)} \cdot \delta f_{(G \cdot C)} - \Delta\epsilon_{(A \cdot U)} \cdot \delta f_{(A \cdot U)}] \quad (13)$$

Denaturation at constant acidic pH. The increment in $\Delta\epsilon_{280}$ due to the denaturation of G·C base-pairs is enhanced when the pH is brought within the range where cytosine residues ionize, because the increase in the degree of ionization of cytosine residues that occurs on denaturation also leads to an increase in ϵ_{280} and the two effects reinforce one another. This effect was described in detail previously (Cox, 1966a,b).

Comparison of the properties of the single-stranded 'stacked' and partly double-helical conformations. As discussed previously (Cox & Kanagalingam, 1967b, 1968) the properties of these two conformations differ in at least three respects. (i) The thermal stability of partly double-helical structures increases as the 1:1 electrolyte concentration is increased from 1mM to 1M, whereas the thermal stability of residues that 'stack' in single-stranded regions is scarcely affected. (ii) The thermodynamics of the equilibrium between the amorphous and stacked forms of adjacent base-residues is the same as for the corresponding dinucleotide i.e. the reaction is not co-operative and increasing the temperature gradually decreases the proportion of 'stacked' residues. However, the transition between the partly double-helical and single-stranded conformations is co-operative, i.e. the standard entropy and enthalpy depends on N , the number of base-pairs/double-helical segment. Consequently the transition between the forms takes place abruptly on altering the environment. (iii) Arising from (ii), the extent of 'stacking' in single-stranded regions is scarcely dependent on the chain length of the polynucleotide, whereas random hydrolysis of diesterified ribose phosphate linkages markedly alters the partly double-helical character of a polynucleotide (for discussion see Cox, 1968b; Cox *et al.* 1968; Cox & Kanagalingam, 1968).

These differences were exploited in the present work.

RESULTS

The reproducibility of the difference spectra and the accuracy of the measurements were discussed previously (Cox & Kanagalingam, 1967a), when it was shown that the composition of a solution containing oligoadenylate and oligoguanylate could be accurately analysed spectrophotometrically. In the present study six independent measurements were made of the extinction of RNA from the smaller ribosomal subparticle (solvent 0.01M-sodium phosphate buffer, pH7.6) at temperatures in the range 25–95°C. The extreme value of $\epsilon_{(P)}$ at a given temperature differed by no more than 100 units at 260nm and by no more than 60 units at 280nm.

In preliminary experiments it was shown that on heating from 25°C to 95°C during the determination of the 'melting' profile limited hydrolysis took place, and the values of $s_{20,w}$ on cooling were

Table 1. Value of $\epsilon_{(P)}$ at 260 nm of rabbit reticulocyte rRNA in neutral, acidic and alkaline solutions

Solvent	pH	$10^{-3} \epsilon_{(P)}$					
		RNA from smaller subparticle			RNA from larger subparticle		
		25°C	95°C	Alkali hydrolysate	25°C	95°C	Alkali hydrolysate
5 mM-Sodium phosphate buffer	3	8.6	9.3	10.6	8.4	9.3	10.2
0.01 M-Sodium phosphate buffer	3	8.0	8.8	10.6	8.0	9.3	10.2
	7	7.6	9.65	11.0	7.75	9.75	10.7
	12	8.8	9.2	10.5	9.15	9.4	10.3
8 M-Urea-0.1 M-sodium phosphate buffer	3	—	—	10.6	9.15	9.3	10.2
	7	—	—	11.0	8.5*	9.75	10.7
	12	—	—	10.5	9.35	9.4	10.3

* Part of the double-helical secondary structure 'melts' before 25°C in this solvent (8 M-urea in 0.1 M-sodium phosphate buffer, pH 7).

10–12S. The original value of ϵ_{260} was regained to within 1% on cooling. The reversibility was checked after each experiment. The size of the fragments found at the end of the heating cycle was scarcely increased when the solution was made 0.1% with respect to sodium dodecyl sulphate to inhibit ribonuclease (this concentration of detergent was shown to be sufficient to inhibit 2 ng of ribonuclease per 50 μ g of RNA/ml), or by lowering the pH of the buffer to 5.5 (a procedure that should decrease the rate of a base-catalysed hydrolysis). I conclude that the extent of hydrolysis described above is unavoidable because of the lengthy (3 h) heating cycle. The observed hydrolysis is apparently much less than that expected on the basis of the work of Eigner, Boedtke & Michaels (1961). The effect of the observed limited hydrolysis on the 'melting' profile has been neglected, since it was estimated from the statistics of random hydrolysis of linear polymers that fragments of size 10–12S would be capable of re-forming 92% of the original hairpin loops of 40 residues on cooling and a higher percentage of smaller loops.

In this study the parameters measured included (i) the 'melting' range (i.e. the temperature range over which changes in secondary structure take place); (ii) the breadth of the 'melting' range that is gauged from the interquartile range (i.e. the temperature range over which ϵ changes from 0.25 to 0.75 of the total increment found on heating from 25°C to 95°C); and (iii) the difference in the spectrum of a heated sample of RNA and another kept at 25°C (measurements of $\Delta\epsilon_{260}$, $\Delta\epsilon_{280}$, and $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ were of particular value). In addition it is also of interest to distinguish changes in the spectrum owing to alterations in the degree of

'stacking' in single-stranded regions from changes owing to 'melting' of double-helical structure.

RNA from the smaller subparticle. The spectrum of RNA (15–20 μ g/ml) from the smaller subparticle was measured in 0.01 M-sodium phosphate buffer, pH 7.6, at 25°C and 95°C and after hydrolysis to nucleotides, and values of $\epsilon_{(P)}$ at 260 nm are given in Table 1. Evidently at 95°C $\epsilon_{(P)}$ is still less than the value found for the alkali hydrolysate. However, as discussed below, there is little organized secondary structure at this temperature.

The difference between the spectrum of RNA at a particular temperature and that of a reference sample kept at 25°C was measured. In addition to 0.01 M-sodium phosphate buffer, pH 7.6, other solvents used were 0.1 M-sodium phosphate buffer, pH 7.6, 1% formaldehyde in 0.1 M-sodium phosphate buffer, pH 7.6, and 4 M-guanidinium chloride in 0.01–0.2 M-sodium phosphate buffer, pH 7.6. Representative results are given in Fig. 1.

The dependence of $\epsilon_{(P)}$ at 260 nm and 280 nm on temperature calculated from Fig. 1(b) and other similar difference spectra are given in Fig. 2. Approximate values of $\epsilon_{(P)}$ for the entirely single-stranded but 'stacked' form of RNA that are required for the interpretation of the difference spectra were obtained by measuring the hypochromism of the formaldehyde derivative (Fig. 2b). RNA was dissolved in 1% formaldehyde-0.1 M-sodium phosphate buffer, pH 7.6. The solution was heated to 95°C to complete the reaction ($\epsilon_{(P)}$ increased from 7600 to 9900) and so prevent the formation of double-helical secondary structure on cooling (Stollar & Grossman, 1962), cooled and then heated for a second time. The increments in $\epsilon_{(P)}^{260}$ and $\epsilon_{(P)}^{280}$ were similar, namely $\Delta\epsilon_{260} = 850$,

Fig. 1. Spectra and difference spectra obtained on heating RNA (19S) from the smaller subparticle. (a) (A) Spectrum of alkaline hydrolysate; (B) spectrum of RNA at 99°C; (C) spectrum of RNA at 22°C (solvent 0.01M-sodium phosphate buffer, pH7.0, in each case). (b) Difference spectra obtained on heating in 0.01M-sodium phosphate buffer, pH7.0 (ϵ_{260} 1.4 at 25°C). (c) Difference spectra obtained at 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 87.5 and 95°C in 4M-guanidinium chloride in 0.01–0.2M-sodium phosphate buffer, pH7.2 (ϵ_{260} 0.880 at 25°C). No correction was applied for changes in the concentration of RNA due to the thermal expansion of water.

$\Delta\epsilon_{280} = 680$ ($\Delta\epsilon_{(P)}^{260}/\Delta\epsilon_{(P)}^{280} = 1.25$), and the ratio $\epsilon_{(P)}^{260}/\Delta\epsilon_{(P)}^{280}$ did not vary appreciably with temperature. The dependence of $\epsilon_{(P)}^{260}$ (after correction for

changes due to hydroxymethylation) on temperature is apparent from Fig. 2(b), which shows that a limiting value is approached at elevated temperatures. At 95°C $\epsilon_{(P)}$ was found to be 9600 at 260nm, which appears to be within 200 units of the limiting value. It is inferred that ϵ_{am}^{260} is within the range 9600–9800, since $\epsilon_{(P)}^{260}$ is scarcely dependent on temperature above 95°C. Hence it is estimated by means of eqn. (2) that in the entirely single-stranded form the fraction, f_s , of base-residues that are 'stacked' at 25°C is 0.25–0.35 and that f_s decreases to 0.0–0.10 at 95°C. It was shown previously (Cox & Kanagalingam, 1968) that about 80% or more of the hypochromism of the entirely single-stranded conformation is found in 1% formaldehyde after heating to 95°C, and that the form of the difference spectra is not altered appreciably by reaction with formaldehyde.

The increment in $\epsilon_{(P)}$ found on heating RNA from the smaller subparticle in 0.01M-sodium phosphate buffer, pH7.6 (Fig. 2c), is much greater than that found for the entirely single-stranded form and is attributed to the 'melting' of partly double-helical structures. The transition approximates to a change from a structure in which a fraction 0.65–0.75 of the residues are base-paired and 0.07–0.15 of the residues are 'stacked' in single-stranded regions at 25°C to one in which a fraction 0–0.10 of the residues are 'stacked' at 95°C. Above 45°C for the entirely single-stranded form the shape of the 'melting' profile ($\Delta\epsilon_{260}/\Delta T$) is 9.1/°C or less compared with 33.6/°C found on 'melting' of the partly double-helical form. The shape of the difference spectrum was found to depend on the temperature range studied. Thus $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ varied from 1.7 to 1.1 for the first and last 'melting' quartiles. This was reflected in the value of T_m calculated from $\Delta\epsilon_{280}$, which was found to be higher than that obtained from $\Delta\epsilon_{260}$ (see Table 2).

When the concentration of sodium phosphate buffer was increased from 0.01M to 0.1M much the same changes in the spectrum were observed, although T_m was increased by about 12°C (Fig. 2a), which compares with a shift in T_m of 14°C found for DNA from rat liver (Cox & Kanagalingam, 1968). The influence of the electrolyte concentration on T_m (see Fig. 3a) is consistent with the notion that changes in double-helical secondary structure make a large contribution to $\Delta\epsilon$.

The denaturation spectrum was also measured when 4M-guanidinium chloride–0.01M-sodium phosphate buffer, pH7.2, was the solvent (Fig. 2d). In this solvent the tendency of base-residues to stack one upon another is decreased by about 60%. For example, it was found for fragments of rRNA having an entirely single-stranded conformation in 0.1M-potassium phosphate buffer that ϵ_{260} increased from 1.04 at 40°C to about 1.10 at 95°C, whereas

Fig. 2. Dependence of ϵ_p^{260} and ϵ_p^{280} of RNA from the smaller ribosomal subparticle on temperature: $\circ$, $\Delta\epsilon_{260}$; $\bullet$, $\Delta\epsilon_{280}$. Solvent: (a) 0.1M-sodium phosphate buffer, pH 7.0; (b) 0.1M-sodium phosphate buffer (pH 7.0)–1% formaldehyde (second and subsequent heating cycles); (c) 0.01M-sodium phosphate buffer, pH 7.0; (d) 4M-guanidinium chloride in 0.01M-sodium phosphate buffer, pH 7.2.

an increase from 1.01 at 40°C to 1.035 at 95°C was obtained when 4M-guanidinium chloride–0.01M-sodium phosphate buffer, pH 7.2, was the solvent (Cox & Kanagalingam, 1968). In the latter solvent the limiting value of ϵ_{am} is readily calculated. In 4M-guanidinium chloride–0.01M-sodium phosphate

buffer, pH 7.2, the T_m of DNA was found to be about 5°C less than in 0.1M-sodium phosphate buffer.

The changes in the spectrum found on heating RNA from the smaller subparticle in 4M-guanidinium chloride–0.01M-sodium phosphate buffer,

Fig. 3. Effect of ionic strength on the denaturation spectrum of rRNA. (a) RNA from the smaller subparticle. The dependence of $\epsilon_{(260)}^{260}$ on temperature for: 1 (○), RNA in the single-stranded form obtained by heating to 95°C in 1% formaldehyde–0.1 M-sodium phosphate buffer, pH 7.0 (cf. Cox & Kanagalingam, 1968); 2 (●), 0.01 M-sodium phosphate buffer, pH 7.0; 3 (△), 0.1 M-sodium phosphate buffer, pH 7.0. (b) RNA from the larger subparticle. The dependence of $\epsilon_{(260)}^{260}$ on temperature for: 1 (○), RNA in the single-stranded ‘stacked’ form obtained by heating to 95°C in 1% formaldehyde–0.1 M-sodium phosphate buffer, pH 7.0 (cf. Cox & Kanagalingam, 1968); 2 (●), 0.01 M-sodium phosphate buffer, pH 7.0; 3 (△), 0.1 M-sodium phosphate buffer, pH 7.0.

Table 2. Summary of the ‘melting’ profile of rabbit reticulocyte rRNA

Solvent	RNA from the smaller subparticle				RNA from the larger subparticle			
	Inter-quartile range (°C)	T_m (°C)		$\Delta\epsilon_{260}/\Delta\epsilon_{280}$	Inter-quartile range (°C)	T_m (°C)		$\Delta\epsilon_{260}/\Delta\epsilon_{280}$
		260 nm	280 nm			260 nm	280 nm	
0.01 M-Sodium phosphate buffer, pH 7.6	33–53	45	52	1.47	47–69	57	68	1.13
0.1 M-Sodium phosphate buffer, pH 7.6	47–65	58	63	1.52	61–82	67	63	1.23
0.1 M-Sodium phosphate buffer, pH 3.7	43–68	54	56	1.19	46–78	62	63	0.97
4 M-Guanidinium chloride–0.01 M-sodium phosphate buffer, pH 7.2	45–65	54	58	1.23	50–80	62	68	0.94

pH 7.2, agree with the ‘melting’ of double-helical secondary structure. Thus, T_m was close to that found in 0.1 M-sodium phosphate buffer, but the

beginning and end of the transition were more clearly defined because, as expected, changes in extinction due to ‘stacking’ in single-stranded

Table 3. Values of $\epsilon_{(P)}$ at 260 nm used in analysing spectrophotometric data

	$\epsilon_{(P)}$ at 260 nm			
	RNA from smaller subparticle		RNA from larger subparticle	
	0.01 M-Phosphate buffer, pH 7.0	4 M-Guanidinium chloride, pH 7.2	0.01 M-Phosphate buffer, pH 7.0	4 M-Guanidinium chloride, pH 7.2
ϵ_{am}^0	9650–9800	9650–9800	9750–10000	9750–10000
ϵ_{dh}^0 (eqn. 12)	6950–7220	7130–7380	7300–7550	7400–7600
(eqn. 12a)	7300		7420	
ϵ_s at 25°C	88000±100	9300±100	8700±100	9400±100
$\epsilon_{obs.}$ at 25°C	7600±100	7980±100	7750±100	8100±100

regions were decreased. The values of $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ were found to depend on the temperature range (Fig. 4). In general $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ was slightly less than the values calculated from the corresponding region of the 'melting' profile obtained in phosphate buffer solutions. This difference is attributed partly to a small perturbation of the spectrum in 4M-guanidinium chloride-sodium phosphate buffer, pH 7.2, and partly to the much smaller contribution to $\Delta\epsilon$ from residues that are 'stacked' in single-stranded regions. As in 0.01M- and 0.1M-sodium phosphate buffer $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ increased as the temperature increased.

The difference spectra were analysed by the methods described in the Theoretical section by using the values of $\epsilon_{(P)}$ given in Table 3. Independently-'melting' double-helical segments of greater than about eight base-pairs were not detected. Values of N were estimated by means of eqn. (5) to lie within the range 3–8 (the ranges given are for $b = 3$ and $b = 20$, and for an error of $\pm 10^\circ\text{K}$ in $T_{m(\infty)} - T_m$). The calculated values of N were similar irrespective of whether eqn. (2) or eqn. (5) was used; the values yielded by eqn. (2) were the same as those obtained from eqn. (5) when $b = 1$. It is unlikely that the calculated values are grossly in error when N is small (<10) because of the dominance of the simple size effect (see Table 5). Eqn. (5) takes into account the average nucleotide composition of the double-helical segments and also the number of unpaired residues within the loop. It is likely that the population of segments 'melting' over a particular temperature range is heterogeneous and that for a particular species of segment having the same overall nucleotide composition the precise nucleotide sequence might affect T_m (Bautz & Bautz, 1964). These are likely to be second-order effects because the dominating factor is N (the number of base-pairs/independently-'melting' double-helical segment). The limiting values of N were calculated on the basis of an error of $\pm 10^\circ\text{K}$ in $T_{m(\infty)} - T_m$. This allowance of $\pm 10^\circ\text{K}$ is equivalent to assigning an additional

range of 20°K in T_m to account for the secondary effects such as those mentioned above and is also greater than the probable interquartile melting range of a single species of double-helical segment.

The considerable overlap in the 'melting' range of RNA from the smaller subparticle with that of unfractionated *Escherichia coli* tRNA (Fig. 4 and Table 5) lends support to the calculated values of N . Since the double-helical segments of these two RNA species are similar in nucleotide composition it is probable that the double-helical segments are comparable in length (four to eight base-pairs in each case). However, the profile of the rRNA species is biased towards higher temperatures, showing that the proportion of higher-'melting' species is greater. It is inferred that in this instance N is probably biased towards 8 rather than towards 4.

The fraction of residues involved in base-pairing at 25°C was estimated to be 0.61–0.69. The higher limit was obtained by decreasing $\epsilon_{am}^0 - \epsilon_{dh}^0$ by 10% to allow for end effects. The content of G·C base-pairs of a double-helical segment varied from 50 to 65%, depending on T_m ; of the total residues forming base-pairs 58% were G·C.

The chain length of RNA from the smaller ribosomal subparticle is about 1500 nucleotides. A fraction (about 0.12) of the nucleotides, i.e. about 180 residues, form double-helical segments, which melt between 40°C and 50°C (solvent 4M-guanidinium chloride–0.01M-sodium phosphate buffer, pH 7.2). Each double-helical segment is 5 ± 2 base-pairs long on average [calculated by means of eqn. (5) when $b = 20$]. Hence it is calculated that there are about 18 (the lower limit is 13, calculated for $N = 7$, and the upper limit is 30, calculated for $N = 3$) double-helical segments with these 'melting' properties. For the whole 'melting' range it was calculated that there are about 64 independently 'melting' double-helical segments/1500 nucleotides; this is a rough estimate limited by the accuracy of values of N . These results, which are summarized in Tables 4 and 5, were calculated from changes in

Fig. 4. Interpretation of the denaturation spectrum of rRNA and *E. coli* tRNA. $\Delta\epsilon/\Delta T$ was plotted against T for (a) RNA from the smaller subparticle, (b) RNA from the larger subparticle and (c) *E. coli* tRNA. $\Delta\epsilon_{260}/\Delta T$ is shown as $\equiv$; $\Delta\epsilon_{280}/\Delta T$, $\parallel$. (G·C) contents (%) were calculated from (a), (b) and (c) by means of eqn. (11), and are shown as parts (d), (e) and (f) respectively. $\Delta f_{dh}/\Delta T$ for (g) RNA from the smaller subparticle, (h) RNA from the larger subparticle and (i) *E. coli* tRNA. $\Delta f_{(G\cdot C)}/\Delta T$, $\equiv$; $\Delta f_{(A\cdot U)}/\Delta T$, $\parallel$; $\Delta f_{dh}/\Delta T$, —. The solvent was 4M-guanidinium chloride–0.01M-phosphate buffer, pH 7.2.

the spectrum of RNA in the three solvents 0.01M-sodium phosphate buffer, pH 7.6, 0.1M-sodium phosphate buffer, pH 7.6, and 4M-guanidinium chloride–0.01M sodium phosphate buffer, pH 7.2. Essentially the same values of f_{dh} , N etc. were obtained irrespective of solvent. Less reliable results

are obtained when 'stacking' is neglected (cf. Gould & Simpkins, 1969).

RNA from the larger subparticle. When RNA from the larger subparticle was studied (see Figs. 3b, 5 and 6) the major differences were found to be (see Table 1): (1) that double-helical secondary

Table 4. *Summary of properties of rabbit reticulocyte rRNA*

Property	Solvent	RNA from smaller subparticle	RNA from larger subparticle
Nucleotide composition (mol %) (Gould, Arnstein & Cox, 1966)	A	22.24	16.6
	U	21.22	15.8
	G	32.17	37.16
	C	24.34	30.5
(A + U)/(G + C) ratio		0.75	0.46
(G + C)/(A + U + G + C) ratio		0.58	0.69
Fraction of residues forming base-pairs at 25°C (f_{dh}) calculated from eqn. (8)	4 M-Guanidinium chloride–0.01 M-sodium phosphate buffer, pH 7.2	0.61–0.69	0.65–0.72
Number (N) of base-pairs/double-helical segment estimated by eqn. (5)		3–10	3–17
Nucleotide composition of double-helical segments [$f_{(G\cdot C)}/(f_{(G\cdot C)} + f_{(A\cdot U)})$] calculated by eqns. (9) or (11) for first and last quartiles	0.01 M-Sodium phosphate buffer, pH 7.6	0.5–0.7 (0.58 overall)	0.5–0.9 (0.68 overall)
	4 M-Guanidinium chloride–0.01 M-sodium phosphate buffer, pH 7.2	0.5–0.65 (0.58 overall)	0.5–0.8 (0.68 overall)
Average no. of residues per hairpin loop ($2N_1 + b$) (see Cox <i>et al.</i> 1968)		25 ± 7	30 ± 6–35 ± 7
Estimated no. of hairpin loops/molecule		40–60	80–120
Estimated no. of double-helical segments/molecule		42–71	170–294

structure has increased thermal stability (i.e. T_m is higher); (2) that the 'melting' range is broader (e.g. in 4 M-guanidinium chloride–0.01 M-sodium phosphate buffer, pH 7.2, the interquartile range was found to be 50–80°C compared with the range 45–65°C noted for RNA from the smaller subparticle); (3) that the difference spectra (Fig. 5) are characterized by a smaller value of the ratio $\Delta\epsilon_{260}/\Delta\epsilon_{280}$, indicating that a higher proportion of G·C base-pairs contribute to the double-helical secondary structure of RNA from the larger subparticle compared with RNA from the smaller subparticle. Values of the $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ ratio were calculated by means of eqn. (9) on the basis of the assumption that $\delta f_{(A\cdot U)}/\delta f_{(G\cdot C)} = (A+U)/(G+C)$. For RNA from the smaller and the larger subparticles respectively, the calculated (1.5 and 1.14) and observed values (1.5 and 1.13; see Table 1) are in good agreement, and support the view that the overall nucleotide composition of the double-helical segments is related to the overall nucleotide composition. However, individual double-helical segments are short enough (10–20 nucleotides compared with 1500 or 4500 nucleotides) to differ one from another in their 'melting' properties as a result of variations in the number of base-pairs and chemical composition of double-helical segments. This is apparent from the variation in $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ with temperature and the breadth of the 'melting' range. It was estimated that there are about 220 independently 'melting' double-helical segments and almost all

appear to be 11 or fewer base-pairs long, and about half are estimated to be no longer than five base-pairs. The fraction of residues forming base-pairs at 25°C was found to be within the range 0.65–0.72 compared with 0.61–0.69 for RNA from the smaller subparticle and 0.6 ± 0.05 for unfractionated tRNA. The nucleotide composition of the double-helical segments appears to vary from about 55% to about 80% G·C base-pairs (see Fig. 4 and Tables 4 and 5), depending on the 'melting' range. Of all the base-paired residues about 69% form G·C base-pairs.

Denaturation by pH-titration at constant temperature. Double-helical secondary structure stabilized by complementary base-pairs is eliminated on titration to the extremes of pH because the $NH_{(1)}-CO_{(6)}$ group ($pK > 7$) of guanine or uracil residues interacts with the $N_{(1)}=C_{(6)}-NH_2$ group ($pK < 7$) of cytosine or adenine residues (for discussion see Cox, 1966b) and the polynucleotide will revert to a single-stranded form with residues 'stacked' to an extent governed by pH and temperature (see Scheme 1). It is evident from Table 1 (cf. Cox & Littauer, 1963; Cox & Arnstein, 1963) that RNA from the smaller subparticles and RNA from the larger subparticles are both hypochromic at the extremes of pH. This hypochromism is attributed to 'stacking' in the single-stranded form. Since 'stacking' is essentially non-co-operative (e.g. Brahms *et al.* 1966) dinucleotides are suitable model compounds. Schemes for the pH titration of dinucleotides given previously (Cox, 1966b) show that changing the pH of

Table 5. *Properties of rabbit reticulocyte rRNA and unfractionated E. coli tRNA deduced from spectrophotometric data (solvent 4 M-quanidium chloride-0.01M-sodium phosphate buffer, pH 7.2)*

RNA species	'Melting' range (°C)	T_m (°K)	(G·C) content (%)	$10^{-3}\Delta(1/T)$	N		$2N+b$ (calc. from alkali hydrolysis)	Δf_{dn}	n_{dn} (based on mean values of N , $b=20$; range calc. for extreme values of N in parentheses)
RNA from the smaller subparticle (chain length about 1500 nucleotides)	30-40	308	47	0.471	$b=3$	$b=20$	25±5	0.06	11
	40-50	318	50	0.389	4±1	5±2	25±5	0.12	18 (13-30)
	50-60	328	50	0.293	5±2	7±2	25±5	0.14	15 (12-21)
	60-70	338	67	0.295	5±2	7±2	25±5	0.12	12 (10-18)
	70-80	348	67	0.210	6±2	8±2	25±5	0.09	8 (7-11)
								Total =	64 (42-71)
RNA from the larger subparticle (chain length about 4500 nucleotides)	30-40	308	55	0.517	3	4	30±6	0.05	25
	40-50	318	56	0.421	4±1	5±2	30±6	0.08	38 (26-60)
	50-60	328	62	0.369	4±1	6±2	30±6	0.12	52 (34-68)
	60-70	338	68	0.299	5±2	6±2	30±6	0.11	42 (31-62)
	70-80	348	78	0.266	6±2	8±2	35±7	0.10	38 (23-38)
	80-90	358	78	0.185	10±3	12±5	35±7	0.09	18 (19-29)
	90-100	368	78	0.106	15	19	35±7	0.04	6
								Total =	219 (170-294)
Unfractionated <i>E. coli</i> tRNA (chain length about 80 nucleotides)	30-40	308	55	0.517	3	4±1	—	0.05	0.5 (0.4-0.66)
	40-50	318	55	0.415	4±1	5±2	—	0.14	1 (0.8-1.8)
	50-60	328	63	0.327	4±1	5±2	—	0.19	1.5 (1.1-2.5)
	60-70	338	73	0.198	4±1	6±2	—	0.15	1 (0.75-1.5)
	70-80	348	65	0.117	8±2	10±3	—	0.08	0.5 (0.25-0.5)
								Total =	4.5 (3.4-6.9)

Fig. 5. Spectra and the difference spectra obtained on heating RNA (30S) from the larger subparticle. (a) Spectra at 22°C (C), 99°C (B) and alkali hydrolysate (A) in 0.01M-sodium phosphate buffer, pH 7.0. (b) Difference spectra measured at 5°C intervals from 25°C, in 0.01M-sodium phosphate buffer, pH 7.0 (E_{260} 1.4 at 25°C). (c) Difference between the reference solutions at 25°C and the sample at 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 87.5, and 95°C in 4M-guanidinium chloride-0.01M-sodium phosphate buffer, pH 7.2 (E_{260} 1.460 at 25°C).

a solution will not affect 'stacking' except within the pH range over which the titratable groups become ionized. Thus the hypochromism of poly A (pK of AMP, 3.7) and poly C (pK of CMP, 4.5) is affected by titration with acid but not with alkali.

Thus in alkaline solutions 'stacking' between adjacent cytosine residues, between neighbouring adenine residues or between neighbouring adenine and cytosine residues should remain the same as in neutral solutions. Guanine and uracil residues became ionized at pH 12.5, so that the tendency of these residues to 'stack' will diminish. In acidic solutions 'stacking' will be diminished when adenine and cytosine residues are involved (for example, titration of adenylyladenylate to pH 3 largely eliminates stacking; R. A. Cox, unpublished work). The pK of guanine residues is low (pK for GMP, 2.4), so that lowering the pH leads first to the elimination of double-helical secondary structure and to precipitation of the polynucleotide before guanine residues have become extensively ionized. Since oligo G may be titrated to pH 3.0 without affecting ϵ_{260} (Cox, 1966b) 'stacking' at pH 3 is probably due to guanine and uracil residues. The addition of urea, which decreases 'stacking' at pH 7, also decreases 'stacking' at the extremes of pH (see Table 1).

Thermal denaturation in acidic solutions. The denaturation of RNA from both the smaller and the larger subparticles in 0.1M-sodium phosphate buffer, pH 3.7, were compared (see Fig. 7). The conclusions derived from the difference spectra obtained at pH 7.0 were confirmed. Comparison of the increments $\Delta\epsilon_{260}$ and $\Delta\epsilon_{280}$ showed that the proportion of G·C base-pairs was greater for the double-helical segments 'melting' at higher temperatures, and the 'biphasic' form of the 'melting' profile was apparent for RNA from the larger subparticle. For reference, the changes in ϵ_{260} and ϵ_{280} for the entirely single-stranded form were obtained after RNA from the larger subparticle was hydrolysed by ribonuclease to fragments of $s_{20\cdot w}$ about 2S, which are too small to form double-helical structures (cf. Cox & Kanagalingam, 1967a, 1968). In this case the ratio $\Delta\epsilon_{280}/\Delta\epsilon_{260}$ decreased as the temperature was raised. Quantitative analysis of this effect is beyond the scope of this paper.

The 'melting' of double-helical segments rich in G·C base-pairs was even more clearly shown when 8M-urea was added to decrease 'stacking' in single-stranded regions. Representative difference spectra for RNA from the larger subparticle (given in Fig. 8) closely approach those found on heating poly (G·C) in acidic solutions, which have the form calculated for the denaturation of G·C base-pairs in acidic solutions (Cox, 1966b).

Unfractionated E. coli tRNA. Because of the dearth of information about the 'melting' of short double-helical segments tRNA was chosen as a reference. It is probable that the form of secondary structure is the same for most species of tRNA (Fresco, Blake & Langridge, 1968). For those

Fig. 6. Dependence of $\epsilon_{(p)}^{260}$ and $\epsilon_{(p)}^{280}$ of RNA from the larger ribosomal subparticle on temperature. $\circ$, $\Delta\epsilon_{260}$; $\bullet$, $\Delta\epsilon_{280}$. Solvents: (a) 0.1 M-sodium phosphate buffer, pH 7.0; (b) 0.1 M-sodium phosphate buffer (pH 7.0)-1% formaldehyde (second and subsequent heating cycles); (c) 0.01 M-sodium phosphate buffer, pH 7.0; (d) 4 M-guanidinium chloride-0.01 M-sodium phosphate buffer, pH 7.2.

species of tRNA of known nucleotide sequence the number of base-pairs/double-helical segment varies from four to eight, and the average size of three-quarters of the hairpin loops ($2N+b$) is about 17 residues (b is about seven residues). A fourth loop encompasses the other three because the molecule in its cloverleaf form is circular. The (A+U)/(G+C) ratio for unfractionated *E. coli* tRNA is intermediate between that of RNA from the smaller and the larger subparticles, the values being respectively 0.58, 0.48 and 0.77. Moreover there is considerable overlap in the 'melting' ranges of these three species of RNA. When the 'melting' profile of tRNA (Fig. 4; see also Cox & Kanagalingam, 1967b) was analysed by the methods

given in the Theoretical section the following values were obtained (see Table 5): $f_{dh} = 0.6 \pm 0.05$; $f_{(A \cdot U)}/f_{(G \cdot C)} = 1.3$; $N = 4-8$; n_{dh} (the number of independently 'melting' double-helical segments/80 nucleotides) = 4.5. These results agree with the secondary structure of tRNA in the cloverleaf form and illustrate the validity of the theoretical analysis.

DISCUSSION

The results of this study confirm and extend work on unfractionated rRNA (Cox, 1966a). The reliability of the information sought, such as the fraction of residues forming base-pairs and the nucleotide composition of double-helical

Fig. 7. Denaturation of rRNA in 0.1M-sodium phosphate buffer, pH 3.7. $\circ$, ΔE_{260} ; $\bullet$, ΔE_{280} . (a) RNA from the smaller subparticle; (b) RNA from the larger subparticle; (c) fragments (1.8S) of RNA from the larger subparticle that do not form double-helical secondary structure. In each case E_{260} 1.0 at 25°C.

Fig. 8. Difference spectra observed on heating RNA from the larger subparticle in 8M-urea, buffered with 0.1M-sodium phosphate. (a) pH 7.0, E_{260} 1.145 at 25°C. The difference spectra, uncorrected for changes in the concentration of RNA due to the thermal expansion of water, were recorded at 25, 40, 50, 60, 70, 80 and 95°C successively. (b) pH 4.3; E_{260} 1.168 at 25°C. The difference curves were obtained at 25, 30, 35, 40, 45 and 50°C successively. (c) pH 3.7; E_{260} = 1.200 at 25°C; otherwise as for (b).

segments, depends on the accuracy of the values of $\Delta\epsilon_{(A\cdot U)}$, $\Delta\epsilon_{(G\cdot C)}$ etc. In this respect this and other optical methods are similar. In all these methods, irrespective of whether optical rotatory dispersion, circular dichroism or the i.r. spectrum is studied, the experimental values are analysed as simple multicomponent systems by using reference data for A·U and G·C base-pairs, due allowance being made for the 'stacked' but unpaired residues. The hypochromic effect over the range 220–300nm was the first to be exploited for analytical purposes because the measurements are easy to make; and there is little doubt that the method gives accurate results when applied to multihelical structures such as native DNA. As with other optical methods the analysis of the partly double-helical structures is more difficult (see the Theoretical section). For this reason it is important that the values obtained by analysis of the hypochromic effect of tRNA should be confirmed by other methods. Thus it was shown (Cox, 1966a) that the pH-titration properties of both *E. coli* and reticulocyte rRNA supported the analysis of the hypochromic effect. The finding that for *E. coli* rRNA G·C base-pairs account for 56% of the base-paired residues was confirmed by analysis of the i.r. spectrum (Cotter & Gratzer, 1969); the estimates for base-paired residues were respectively 70% (neglecting the contribution of 'stacked' but unpaired residues) from u.v. and 60% from i.r. measurements.

The accuracy of the analysis of the hypochromic effect was further tested by studying the properties of double-helical RNA and of unfractionated *E. coli* tRNA. In each case there was good agreement

Fig. 9. Schematic representation of possible conformations of rRNA. (a) A hairpin loop with one double-helical segment that 'melts' to a single-stranded form; (b) a hairpin loop with one double-helical segment that 'melts' in two steps owing to nucleotide sequence; (c) a hairpin loop with two double-helical segments that 'melt' independently because they are separated by non-paired residues. Data for RNA from the larger subparticle (Tables 4 and 5) formed the basis of these illustrations. (b) and (c) are consistent with estimates of the size of the hairpin loops. The base residues are indicated by dashes and hydrogen bonds formed between base residues are shown as dots.

between the properties deduced from the changes in the u.v. spectrum and the known properties of these RNA species. Further information about the 'melting' of single chemically defined hairpin loops may improve the analysis.

It is emphasized that the values of f_{dh} , G·C content (%) and N deduced for double-helical segments 'melting' over a particular temperature range are average values and more than one species of double-helical segment may be involved. More-

over the relation between one species of double-helical segment and another cannot be inferred from the 'melting' profiles. There are at least three possibilities (see Fig. 9): (a) each hairpin loop may comprise one double-helical segment that 'melts' as a single species; (b) although as in (a) there may be one double-helical segment/hairpin loop the nucleotide sequence may be such that regions of the double-helical segment 'melt' independently of one another, leading to a biphasic or multiphasic profile

(for theoretical analysis see Kallenbach, 1968); (c) there may be more than one double-helical segment/hairpin loop, each separated from another by unpaired residues. All of these possibilities might occur in a particular molecule. For RNA from the smaller ribosomal subparticle the data (Table 4) suggests that a high proportion (at least half) of the hairpin loops comprise a single double-helical segment. The 'melting' profile of RNA from the larger subparticle has features consistent with a biphasic transition, i.e. about 25% of residues form double-helical segments of about 56% G+C content that 'melt' below 60°C (solvent 4M-guanidinium chloride-0.01M-sodium phosphate buffer, pH 7.2) and about 25% of residues form double-helical segments of about 80% G+C content that 'melt' above 70°C. Although these observations are consistent with the biphasic 'melting' of a single double-helical segment the other possibilities mentioned above cannot be excluded. Estimates of the size of the hairpin loop (Cox, 1967; Cox *et al.* 1968) based on the statistics of degradation of linear polymers are close to estimates based on the size of crystallizable fragments (Spencer, Pigram & Littlechild, 1969), which are believed to approximate to single hairpin loops of about 40 nucleotides. Thus these results allow the possibility that there might be more than one independently melting double-helical segment/hairpin loop. Further work is required to resolve these possibilities.

EXPERIMENTAL

rRNA. Ribosomes were prepared from the reticulocytes of anaemic rabbits by methods described previously (Arnstein, Cox, Gould & Potter, 1965). The ribosomes were dissociated into subunits with EDTA (Gould *et al.* 1966) and the subunits were separated by zone centrifugation (Klucis & Gould, 1966). RNA was isolated from the subunits by the guanidinium chloride method (Cox, 1966c). Unfractionated *E. coli* tRNA (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).

Spectrophotometry. Absorption spectra were measured on a Unicam SP.700 recording spectrophotometer. Temperature-extinction profiles were measured by using both a Unicam SP.700 recording instrument equipped with an electrically heated cell-block and a Beckman DU manual instrument with a thermostatically controlled cell-housing supplied from a Haake-Fe circulating thermostat. The temperature was measured in the first case with a thermistor in the cell-block, in the second with a mercury thermometer in a separate cell.

Molar residue extinction coefficients, ϵ_{P} , were obtained by dilution of stock solutions of the RNA into 0.4M-KOH and measurement of the spectra after complete hydrolysis. From the base-composition of the RNA (Gould *et al.* 1966) and standard molar extinction coefficients for the nucleotides in alkaline solution (Beavan, Holiday &

Johnson, 1953), molar extinction coefficients could be calculated with good precision.

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