A Study of the Hydrolysis of Unfractionated Reticulocyte Ribosomal Ribonucleic Acid by Pancreatic Ribonuclease and its Relevance to Secondary Structure

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1. Unfractionated RNA from reticulocyte ribosomes was hydrolysed with pancreatic ribonuclease at 25°. The molecular weight decreased rapidly to about 3s when about 6% of the residues were soluble in 0.5N-perchloric acid. In the early stages 60–80% of the hydrolysed linkages were 'hidden'. The denaturation spectrum was affected. Continued hydrolysis led to slow changes in *S* value, in the electrophoresis pattern in polyacrylamide gels and in the denaturation spectrum. 2. Hydrolysis of RNA with alkali to fragments of between 2.8s and 5.9s led to changes in the denaturation spectrum similar to those observed in the early stages of enzymic hydrolysis. 3. A theory was developed to relate changes in secondary structure with main-chain scission. 4. The results agree with the 'hairpin-loop' model for RNA. The denaturation studies are consistent with the presence of more than one species of hairpin loop that differ in their denaturation spectra. The average length of the hairpin loop was estimated to be 10–20 residues and an upper limit of 35 residues was established. 5. It is inferred, on the basis of studies with model compounds, that the stability of single-stranded stacked structures is hardly dependent on salt concentration. 6. The denaturation spectrum of the fragments obtained on hydrolysis became less dependent on ionic strength, suggesting that double-helical structures revert to a single-stranded stacked form on denaturation.

The secondary structure of ribosomal RNA is relatively weak and is believed to arise from bonds formed between base residues situated on different segments of the same molecule so that the polynucleotide chain is folded back on itself. Ribosomal RNA may therefore be regarded as an assembly of 'hairpin loops' of $2N + b + c$ residues, on average, where N is the number of base pairs, b is the number of residues forming the closed end of the loop and c is the number of residues forming the flexible segments linking one hairpin loop to another. The hairpin-loop model was first proposed by Doty, Boedtker, Fresco, Haselkorn & Litt (1959), and, although subsequent work has amply confirmed the existence of organized secondary structure (for review see Spirin, 1963), there has been slow progress in formulating a more precise picture of ribosomal RNA. However, the presence of double-helical segments has been confirmed by X-ray-diffraction studies (Spencer, Fuller, Wilkins & Brown, 1962) and the length of the rod-like segments has been estimated to be 50–150 Å from measurements of mass/unit length (Timasheff, Witz & Luzzatti, 1961). Estimates of the helical content

$[2N/(2N + b + c) \simeq 0.7]$ have been made on the basis of the assumption that only the $2N$ residues of the double-helical segments have decreased chemical reactivity towards acid, alkali, formaldehyde etc., or a reduced extinction coefficient ($\epsilon_{(P)}$) or increased optical rotatory dispersion compared with mononucleotides (Spirin, 1963). An additional assumption, namely that the polynucleotide reverts to an amorphous form when the double-helical segments denature, was introduced in estimating N to be within the range 4–17 base pairs from an analysis of the denaturation spectrum (Cox, 1966*a,b*). Assumptions of this nature may be approximate, since studies with model compounds have established that residues may stack one upon another along a single polynucleotide chain and so lead to a lower $\epsilon_{(P)}$ value (Brahms, Michelson & Van Holde, 1966; Leng & Felsenfeld, 1966). The tendency of base residues to have preferred orientations along a single polynucleotide chain does not depend appreciably on chain length, in contrast with double-helical secondary structure.

The possibility that degradation studies might provide further information about secondary

structure was therefore considered. RNA from reticulocyte ribosomes was hydrolysed by ribonuclease or by alkali. To provide a frame of reference a theory was developed to relate changes in molecular weight and in secondary structure to the probability of hydrolytic cleavage. The results obtained were found to be in general agreement with the hairpin-loop model.

THEORETICAL

An average double-helical segment ABCDEF is represented in Fig. 1. The average number of residues between one segment and the next is c , there are N base pairs (ABDE), and b nucleotides form the closed loop (BCD).

We assume that the stability of the double-helical segment is governed by N , and its nucleotide composition

(Doty, 1961; Lipsett, Heppel & Bradley, 1961; Lipsett, 1964), and that a minimum number, N^0 , of base pairs is required to stabilize a double-helical segment at 25°, the precise value depending on the proportion of A-U* and G-C base pairs. However, the limiting value of N^0 for RNA is probably in the range 3-6, the values of N^0 for G-C and A-U model compounds respectively. Formally, the

*Abbreviations: A, C, G and U, adenine, cytosine, guanine and uracil residues 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., oligoadenylic acid etc.; poly(A+U), complex formed between equimolar amounts of polyA and polyU; polyAU, copolymer of AMP and UMP; s-RNA, 'soluble' or transfer RNA.

Fig. 1. Possible effects of enzymic hydrolysis of RNA. (a) Hypothetical double-helical segment of RNA; N is the number of base pairs, b is the number of residues forming the closed end BCD of the loop, and c residues are found between one double-helical segment and the next. N^0 is the minimum number of base pairs required to stabilize the double-helical structure at 25°. (b) Single-stranded (denatured) form of (a) obtained on increasing the temperature. The transition is reversible when the phosphodiester linkages remain intact. Treatment of (a) with ribonuclease may lead to main-chain scission (indicated by X) at four different sites. (c) Chain scission at site (1), within the region EF, will lead to an immediate decrease in molecular weight (d); the double-helical segment is not affected and will denature reversibly (d) and (e). Scission at site (2) in (c), i.e. of bonds in the region APQE less than N^0 residues from AB, will also lead to an immediate decrease in molecular weight (f). Although the double-helical segment is decreased in chain length, the fragment will denature reversibly (g). If N is reduced to less than 20 denaturation will be observed at a lower temperature range. Chain scission at site (3) of phosphodiester bonds in the region PBDQ further than N^0 residues from AB will immediately affect double-helical secondary structure, but not the apparent molecular weight (h). Denaturation will be observed at lower temperatures if N is reduced to less than about 20. Once denaturation is achieved the fragments separate (i) and will be unlikely to recombine on cooling, so that the effect is not reversible. The molecular weight will be decreased. Chain scission at site (4) in (c), i.e. in the region BCD, will have no apparent effect on either the molecular weight or double-helical secondary structure at 25° (j). Once the double-helical segment unfolds (e) the fragments will separate, a decrease in molecular weight will be observed and denaturation will be irreversible. The temperature range over which denaturation is observed will remain unchanged because N is not affected.

heat of hydrolysis of the phosphodiester bond is itself sufficient to 'melt' N^B base pairs.

Let L^0 be the total number of phosphodiester linkages, H be the number of hydrolysed linkages, and H_{EF} , H_{dh} and H_{BCD} respectively be the numbers of hydrolysed linkages within the regions specified by the suffix, i.e. EF, ABDE and BCD of Fig. 1. Hence:

$$H = H_{EF} + H_{dh} + H_{BCD}$$

and

$$\frac{H}{L^0} = \frac{H_{EF}}{L^0} + \frac{H_{dh}}{L^0} + \frac{H_{BCD}}{L^0} \quad (1)$$

where s_{EF} etc. are coefficients that are functions of $\bar{p}$ introduced to account for the possible non-random distribution of bonds differing from the average sensitivity of the pyrimidine 3'-nucleoside bond to hydrolysis.

The principal possible effects of hydrolysis on the double-helical segment (see Fig. 1a) may now be enumerated.

(1) *Hydrolysis leading to an immediate decrease in molecular weight without affecting double-helical secondary structure.* This effect will be observed (Figs. 1d and 1e) if a bond is hydrolysed in the region EF (Fig. 1c) joining two hairpin loops. The probability that this effect will be observed is:

$$\bar{p}_{EF} = \frac{c\{f_{EF}[p's'_{EF}x_{EF} + (1-x)p''s''_{EF}]\}\bar{p}}{c\{f_{EF}[x_{EF}p's'_{EF} + (1-x)p''s''_{EF}]\} + 2Nf_{dh}p''s''_{dh} + b\{f_{BCD}[x_{BCD}p's'_{BCD} + (1-x)p''s''_{BCD}]\}} \quad (6)$$

If $\bar{p} = H/L^0$ and $\bar{p}_{EF}$ etc. = H_{EF}/L^0 etc., then:

$$\bar{p} = \bar{p}_{EF} + \bar{p}_{dh} + \bar{p}_{BCD} \quad (2)$$

Let R , R_{EF} etc. be the fractions of the total number of linkages that are sensitive to enzymic hydrolysis, and $f = R/L^0$ and $f_{EF} = R_{EF}/L^0$ etc. Hence $\bar{p}/f$ is an average probability of a sensitive bond being hydrolysed. In the general case it is essential to consider the effects of both

The limiting cases are observed when (i) hydrolysis occurs at random, i.e. $p' = p'' = p''$, and $f_{EF} = f_{dh} = f_{BCD} = f$, and $s'_{EF} = s'_{BCD} = s'_{BCD} = s_{dh}$. Hence:

$$\bar{p}_{EF} = \frac{c\bar{p}}{2N + b + c} \quad (7)$$

and when (ii) double-helical segments are completely unaffected by enzyme, i.e. $p'' = 0$. Hence:

$$\bar{p}_{EF} = \frac{c\{f_{EF}[x_{EF}p's'_{EF} + (1-x)p''s''_{EF}]\}\bar{p}}{c\{f_{EF}[x_{EF}p's'_{EF} + (1-x)p''s''_{EF}]\} + b\{f_{BCD}[x_{BCD}p's'_{BCD} + (1-x)p''s''_{BCD}]\}} \quad (8)$$

nucleotide sequence and secondary structure on the hydrolysis of a nucleotide linkage. The sensitivity of a phosphodiester linkage, N_{1p} , of the dinucleotide N_1pN_2 to hydrolysis by pancreatic ribonuclease depends on the identity of the base residues N_1 and N_2 . For hydrolysis to take place, N_1 must be a pyrimidine, since pyrimidine 3'-nucleosides only are attacked. N_2 , however, influences the rate of hydrolysis (Witzel & Barnard, 1962) and the same effect may have to be considered if the dinucleotide forms part of the longer polynucleotide chain. For a given set of experimental conditions the probability of the bond N_{1p} being hydrolysed is defined as p' , p'' and p'' respectively when N_{1p} is in an amorphous, single-stranded stacked or double-helical environment.

Let x_{EF} and x_{BCD} respectively be the fractions of residues in the regions EF and BCD that have an amorphous conformation. Hence:

$$H_{EF} = c f_{EF} [x_{EF} p' s'_{EF} + (1-x) p'' s''_{EF}] \quad (3)$$

$$H_{dh} = 2N f_{dh} p'' s''_{dh} \quad (4)$$

$$H_{BCD} = b f_{BCD} [x_{BCD} p' s'_{BCD} + (1-x) p'' s''_{BCD}] \quad (5)$$

$$\bar{p}_{EF} = c/(b+c)$$

when $p' = p''$, $f_{EF} = f_{BCD}$, and $s'_{EF} = s'_{EF} = s'_{BCD} = s'_{BCD}$. When $p'' = 0$ then the scissions leading to an immediate decrease in molecular weight can only take place in the region DEF, so that the fragments will be multiples of approx. $(2N+b)$ residues. The weight fraction, W_x , of each molecular-weight species may be calculated from the equation:

$$W_x = x(2N+b)(1-\bar{p}_{EF})^x (\bar{p}_{EF})^2 \quad (9)$$

If $p' = p'' = p''$, then:

$$W_x = x(2N+b) \left(1 - \frac{c\bar{p}}{2N+b+c}\right)^x \left(\frac{\bar{p}}{2N+b+c}\right)^2 \quad (10)$$

A broad spectrum of molecular weights differing in units of approx. $(2N+b)$ is also predicted for the case of random scission if double-helical secondary structure is present.

(2) *Hydrolysis immediately affecting both chain length and secondary structure.* Hydrolysis of any bond within the double-helical segment that is $(N^0 + N^B)$ residues from the 'open' end AE (Figs. 1a and 1b) will have this effect and the chance of this event occurring is:

$$\bar{p}_{dh} = \frac{2(N^0 + N^B) p'' s''_{dh} f_{dh} \bar{p}}{c\{f_{EF}[x_{EF}p's'_{EF} + (1-x)p''s''_{EF}]\} + 2Np''s''_{dh}f_{dh} + b\{f_{BCD}[x_{BCD}p's'_{BCD} + (1-x)p''s''_{BCD}]\}} \quad (11)$$

$\bar{p}'_{dh} = 0$ when $p'' = 0$; and:

$$\bar{p}'_{dh} = \frac{2(N^0 + N^B)\bar{p}}{2N + b + c} \quad (12)$$

if there is random degradation of a random nucleotide sequence.

(3) *Hydrolysis leading to no immediate change in molecular weight but affecting double-helical secondary structure.* The hydrolysis of any bond ($N - N^0 - N^B$) residues from A or E will have this effect (Fig. 1h). The hydrolysis of a linkage y

within the region BCD (Figs. 1a and 1c) will not lead to a decrease in molecular weight because the N base pairs are sufficient to hold the fragments together. The denaturation of the double-helical segment will follow its usual course until the intramolecular bonds are broken (Fig. 1j). Once this occurs the two fragments will separate with negligible chance of recombining (Fig. 1k); the effect of hydrolysis on chain length will then become apparent and the original secondary structure will not re-form, i.e. an irreversible change in the spectrum will be observed. The chance of this event occurring is:

$$\bar{p}_{BCD} = \frac{b\{f_{BCD}[p' s'_{BCD} x_{BCD} + (1-x)p'' s''_{BCD}]\bar{p}}{c\{f_{EF}[x_{EF}p' s'_{EF} + (1-x)p'' s''_{EF}]\} + 2Nf_{dh}p'' s''_{dh} + b\{f_{BCD}[x_{BCD}p' s'_{BCD} + (1-x)p'' s''_{BCD}]\}} \quad (15)$$

residues from the end AE will, on the basis of the work of Doty (1961), Lipsett *et al.* (1961) and Lipsett (1964), lead to two double-helical segments of y and $(N-y)$ base pairs that denature independently according to their chain length and nucleotide composition. There will be an obvious effect on T_m if $(N-y)$ and y are both greater than $(N^0 + N^B)$ but less than about 20 (Doty, 1961; Lipsett, 1964). The denaturation of the newly formed double-helical segments will be observed over a lower temperature range than the intact structure. Once denaturation is complete the fragments will separate and are unlikely to re-form on rapid cooling, so

When degradation takes place at random and the distribution of nucleotides is not significantly different from random, then:

$$\bar{p}_{BCD} = \frac{b\bar{p}}{2N + b + c} \quad (16)$$

Chain scission and 'hidden' breaks

The probability, h , of scissions of the type described in eqns. (3) and (4) that do not immediately affect molecular weight is related to N by the equation:

$$h = \frac{\bar{p}[2(N - N^0 - N^B)p'' f_{dh} s_{dh} + b\{f_{BCD}[x_{BCD}p' s'_{BCD} + (1-x)_{BCD}p'' s''_{BCD}]\}]}{c\{f_{EF}[x_{EF}p' s'_{EF} + (1-x)p'' s''_{EF}]\} + 2Nf_{dh}p'' s''_{dh} + b\{f_{BCD}[x_{BCD}p' s'_{BCD} + (1-x)p'' s''_{BCD}]\}} \quad (17)$$

when $p'' = 0$.

$$h = \frac{b\{f_{BCD}[x_{BCD}p' s'_{BCD} + (1-x)_{BCD}p'' s''_{BCD}]\bar{p}}{c\{f_{EF}[x_{EF}p' s'_{EF} + (1-x)p'' s''_{EF}]\} + b\{f_{BCD}[x_{BCD}p' s'_{BCD} + (1-x)p'' s''_{BCD}]\}} \quad (18)$$

that the 'hidden' break will then be revealed and the original spectrum will not be observed on cooling.

The probability of this event occurring is:

If, in addition, $p' = p''$, and the nucleotide sequence is near-to-random, then:

$$h = \frac{b}{(b+c)}$$

$$\bar{p}''_{dh} = \frac{2(N - N^0 - N^B)p'' s''_{dh} f_{dh} \bar{p}}{c\{f_{EF}[x_{EF}p' s'_{EF} + (1-x)p'' s''_{EF}]\} + 2Np'' s''_{dh} f_{dh} + b\{f_{BCD}[x_{BCD}p' s'_{BCD} + (1-x)p'' s''_{BCD}]\}} \quad (13)$$

For random degradation of a random polynucleotide:

$$\bar{p}''_{dh} = \frac{2(N - N^0 - N^B)\bar{p}}{2N + b + c} \quad (14)$$

and $\bar{p}''_{dh} = 0$ when $p'' = 0$.

(4) *Hydrolysis of linkages that are seen to affect molecular weight and secondary structure once residual double-helical secondary structure is destroyed.* Hydrolysis of a bond

For a random nucleotide sequence that is attacked at random, i.e. when $p' = p'' = p''$ etc.:

$$h = \frac{[2(N - N^0 - N^B) + b]\bar{p}}{2N + b + c} \quad (19)$$

When $p \rightarrow 0$, $h' = h/\bar{p}$ will represent the fraction of 'hidden' breaks that will be revealed once secondary structure is destroyed.

Fig. 2. Relation between the fraction, k' , of 'hidden' breaks in the diesterified ribose phosphate backbone and secondary structure. RNA at 25° is regarded as a hypothetical chain made up, per 1000 residues, of L double-helical segments, each of N base pairs, and a closed loop of b residues with c residues linking one such segment to another (Fig. 1a). The maximum possible percentage, H , of residues forming base pairs will be obtained when both b and c are minimum. This condition is approached when $b=c=4$, and the corresponding values of L and H for a particular value of N are given in (a). In this case k' will have a unique value for each value of N . In principle both H and N may be evaluated, but these two parameters do not completely define secondary structure, which also depends on b and c . The range of possible structures, once H and N are defined, is determined by the limiting value of b and c , both of which cannot be less than 3. (b) When $H=70$, k' is no longer uniquely determined by N . The limiting values of k' were calculated by substituting first $b=4$ and $c=0.86N-4$, and secondly $b=0.86N-4$ and $c=4$, in eqn. (6), so that possible values of k' lie within the area ABC. (c) Values of k' were calculated for $H=50$ (i.e. $2N=b+c$) when $b=4$ and $c=2N-4$, and when $b=2N-4$ and $c=4$: the insets illustrate the extremes of possible structures. The intermediate curve was calculated on the basis of $b=c=N$ in eqn. (6).

A knowledge of h' may serve to distinguish between alternative possible configurations once N and H (the fraction of base residues participating in double-helical secondary structure) are known precisely. Thus:

$$(b+c)/2N = (1-H)/H \quad (20)$$

and by introducing minimum values of b and c respectively (about 3 according to Fresco, Alberts & Doty, 1960) into eqn. (20) it is possible to calculate a range of values for h' when N and H are also specified. A range of values for h' may also be calculated for particular values of N and H when $p''=0$ by substituting minimum values for b and c into eqn. (19).

In Fig. 2(a) h' is given as a function of N when H is maximum (i.e. $b=4$, $c=4$). The values of H and of the number of loops/1000 residues are also given in Fig. 2(a). In Fig. 2(b) values of h' are given as a function of N for when $H=0.7$, the value obtained by Cox & Arnstein (1963) for reticulocyte ribosomal RNA. The observed value of H is likely to be an over- rather than an under-estimate. The values of h' are given as a function of N in Fig. 2(c) when $H=0.5$, assuming $c=4$ and b is maximum (upper curve) and assuming $b=4$ and c is maximum (lower curve). A knowledge of h' ($\bar{p} \rightarrow 0$) may be of value even if degradation does not take place at random, since relative values of b and c may be derived. However, as $\bar{p}$ increases h' will no longer be equal to the fraction of 'hidden' breaks. In the general case, the fraction, f' , of loops that do not have 'hidden' breaks is:

$$f' = \left(1 - \frac{\bar{p}_{dh}}{2N}\right)^{2(N-N^0-N^E)} \left(1 - \frac{\bar{p}_{BCD}}{b}\right)^b \quad (21)$$

Hence the fraction of loops having 'hidden' breaks is:

$$1-f' = 1 - \left(1 - \frac{\bar{p}_{dh}}{2N}\right)^{2(N-N^0-N^E)} \left(1 - \frac{\bar{p}_{BCD}}{b}\right)^b \quad (22)$$

Multiple scissions within the same loop will lead to no more than one 'hidden' break. The first scission will not immediately affect molecular weight, but subsequent scissions will lead to the immediate removal of one or more fragments from within the loop.

Thus the fraction of scissions that are 'hidden' is equal to the number of suitably damaged loops divided by the total number of scissions. The number of loops is $L^0/(2N+b+c)$, and the total number of scissions is $H=L^0\bar{p}$; hence:

$$h' = \frac{(1-f')}{\bar{p}(2N+b+c)} = \frac{1 - \left(1 - \frac{\bar{p}_{dh}}{2N}\right)^{2(N-N^0-N^E)} \left(1 - \frac{\bar{p}_{BCD}}{b}\right)^b}{\bar{p}(2N+b+c)} \quad (23)$$

When $\bar{p}_{dh}/2N = \bar{p}_{BCD}/b$, i.e. degradation is random, then:

$$h' = \frac{1 - (1 - \bar{p})^{(2N+b)}}{\bar{p}(2N+b+c)} \quad (24)$$

When $\bar{p} \rightarrow 0$, eqn. (24) reduces to eqn. (19).

If $\bar{p}_{dh}=0$, i.e. double-helical segments are not attacked, or if $N=N^0$:

$$h' = \frac{1 - \left(1 - \frac{\bar{p}_{BCD}}{b}\right)^b}{\bar{p}(2N+b+c)} \quad (25)$$

Effect of hydrolysis on double-helical secondary structure

Each hairpin loop will make its full and reversible contribution to secondary structure provided that the $(2N+b)$ bonds of the hairpin loop are intact. The fraction, f , of intact loops is:

$$f = \left(1 - \frac{\bar{p}_{dh}}{2N}\right)^{2N} \left(1 - \frac{\bar{p}_{BCD}}{b}\right)^b \quad (26)$$

Hence f is also the fraction of the original secondary structure persisting, provided that the c residues linking one hairpin loop to another do not contribute to secondary structure. When hydrolysis takes place at random, i.e. $\bar{p}_{dh}/2N = \bar{p}_{BCD}/b = \bar{p}$ then:

$$f = (1 - \bar{p})^{(2N+b)} \quad (27)$$

However, if $\bar{p}_{dh}/2N=0$, then:

$$f = \left(1 - \frac{\bar{p}_{BCD}}{b}\right)^b \quad (28)$$

Alkaline hydrolysis. The hydrolysis of RNA by alkali at pH 12 should take place in a near-random fashion, although not all phosphodiester linkages are necessarily hydrolysed at the same rate (Kaltreider & Scott, 1962; Michelson, 1963; Lane & Butler, 1959). The specificity is at least substantially different from that of pancreatic ribonuclease since both purine and pyrimidine 3'-nucleosides are attacked. Moreover, hydrolysis at pH 12 or above takes place when double-helical secondary structure is absent, in contrast with hydrolysis by ribonuclease at pH 7.

Provided that the double-helical secondary structure denatures reversibly, i.e. when the $(2N+b)$ residues of a hairpin remain intact after exposure to high pH, the double-helical structure will re-form on neutralization, then persistence of the original double-helical secondary structure will be related to the probability of chain scission by eqn. (27). The weight distribution of the fragments is given by eqn. (9) by substituting unity for $(2N+b)$.

Experimental approach

To exploit the relationships derived above it is necessary to measure the probability of a bond being hydrolysed, the fraction of 'hidden' breaks and changes in the original secondary structure. The following methods were used.

Probability of a bond being hydrolysed. The probability of a bond being hydrolysed is defined as the number of hydrolysed linkages divided by the total number of linkages present initially. Ideally a method of assaying new end groups is required, but limited digestion by ribonuclease leads to the formation of nucleoside 2',3'-(cyclic)-phosphate derivatives (Markham & Smith, 1952) that are stable between pH 4 and pH 9 (Brown, Magrath & Todd, 1952), as well as 2'- and 3'-phosphates. The latter but not the former may be detected by simple means such as titrimetry.

However, $\bar{p}$ is also equal to the reciprocal of the number-average chain length, L_n . For polydisperse solutions obtained by random degradation of a high-molecular-weight species the number-average molecular weight, M_n , is half the weight-average molecular weight, M_w , as shown, for example, by Oth & Desreux (1954). Hydrolysis by alkali (pH 13) should therefore lead to polydisperse solutions

where $M_n = 0.5M_w$. Polydisperse solutions (where $M_n = 0.5M_w$) should also be obtained on digestion by pancreatic ribonuclease before 'hidden' breaks are revealed even though the predicted increments in molecular weight, namely multiples of $(2N+b)330$ (where 330 is the mean residue weight), are substantially higher than is usual in degradation studies, provided that each of the regions EF has the same probability, $\bar{P}_{EF}$, of suffering main-chain scission. Once 'hidden' breaks are revealed the increments in molecular weight may more closely approach multiples of $0.5(2N+b)330$.

Hall & Doty (1958) studied calf-liver ribosomal RNA that was partly degraded (probably by ribonuclease) during its isolation. These preparations were then briefly heated to 95°. The sedimentation coefficients were then measured in 0.01M-phosphate buffer, pH7, and the molecular weight was established by light-scattering. These two parameters were found to be related by the equation:

$$S_{20,w} = 2.1 \times 10^{-2} M_w^{0.49} \quad (29)$$

The nucleotide composition of calf-liver ribosomal RNA is close to that of reticulocyte ribosomal RNA so that eqn. (29) appears to be relevant to these studies. Equations similar to eqn. (29) have been established for both tobacco-mosaic-virus RNA (Boedtker, 1960) and *Escherichia coli* ribosomal RNA (Kurland, 1960). Hence:

$$L_n = 330/0.5M_w \quad (30)$$

provided that degradation takes place at random.

Extensive hydrolysis may also lead to the formation of fragments of probably not more than four nucleotides maximum (probably two on average) that do not sediment under our experimental conditions. Such fragments are soluble at pH1. Thus, if the fraction of residues soluble at low pH is A , then, on average, the number of fragments is $0.5A$. Hence:

$$\bar{p} = 330/0.5M_w + 0.5A \quad (31)$$

A is small when M_w is about 30000 or more, and the term $0.5A$ becomes appreciable if oligonucleotides are removed from fragments having segments that are resistant to hydrolysis by ribonuclease.

Detection of 'hidden' breaks. 'Hidden' breaks should be revealed once secondary structure is destroyed. Reticulocyte ribosomal RNA in 0.1M-phosphate buffer is largely in the amorphous form at 85°, so that raising the temperature to this value should be sufficient to release the fragments held by double-helical secondary structure. However, thermal hydrolysis of phosphodiester linkages may result from this procedure (Eigner, Boedtker & Michaels, 1961). The procedure finally adopted was to measure the sedimentation coefficient at 10–20° first without any previous treatment and then after heating to 85° for periods from 3 to 30min. A plot of $1/(S_{20,w})^2$ against the time of heating at 85° was then made (cf. Möller & Boedtker, 1961) on the basis of the assumption that eqn. (29) applies. To eliminate the effects of thermal hydrolysis the value obtained on extrapolation to zero time of heating at 85° was used to yield the true molecular weight of the fragments. If $L_{n(85)}$ is the number-average chain length derived in this way and $L_{n(25)}$ is the number-average chain length computed from the sedimentation pattern of RNA before heating, then:

$$h' = (1/L_{n(85)} - 1/L_{n(25)}) / (1/L_{n(85)}) \quad (32)$$

Measurement of changes in secondary structure. The secondary structure of reticulocyte ribosomal RNA was characterized by means of the changes in extinction over the range 220–320m μ with temperature (Cox, 1966a). The precise differences were obtained by measuring the difference spectrum of a solution of RNA in the reference cell maintained at 25° and an equivalent solution heated to a temperature in the range 25–95°. The difference spectrum of neutral solutions of RNA reflects both the number and identity of base pairs reverting to an amorphous form over a particular temperature range (Felsenfeld & Sandeen, 1962; Fresco, Klotz & Richards, 1963) provided that only A–U and G–C base pairs contribute to secondary structure, i.e. other effects such as the stacking of adjacent nucleotides noted even for dinucleotides (Fasman, Lindblow & Grossman, 1964; Brahms *et al.* 1966; Leng & Felsenfeld, 1966) can be neglected. These conditions appear to be largely fulfilled (Cox, 1966a,b) for intact ribosomal RNA. The changes in secondary structure as a result of enzymic hydrolysis were followed by studying changes in the denaturation spectrum.

EXPERIMENTAL

Chemicals. Guanidinium chloride was prepared by neutralization of A.R. guanidinium carbonate with conc. HCl.

Bentonite (Fisher Scientific Co., Fair Lawn, N.J., U.S.A.) was equilibrated with 5mM-MgCl₂–1mM-potassium phosphate buffer, pH7, at 4°, and a fraction sedimenting in the range 6000–15000g for 20min. at 20° was retained.

Pancreatic ribonuclease was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

OligoG, oligoC and oligoA (about six residues in length; see Michelson, 1959) were given by Dr A. M. Michelson.

RNA preparations. RNA was isolated from rabbit reticulocyte polysomes by precipitation as the guanidinium salt (Cox & Arnstein, 1963) and purified by repeated dissolution in and precipitation from guanidinium chloride (Cox, 1966c).

Ultracentrifuging. A Spinco model E ultracentrifuge fitted with absorption optics was used to study the sedimentation behaviour of RNA (about 0.05g./l.) at 10–15°. A 12mm. path-length cell was used in all cases. The sedimenting boundaries were translated into a plot of percentage transmission against distance on an XY recorder by replacing the camera of the producton model with a photomultiplier that scanned the aperture at 4min. intervals. Sedimentation coefficients were calculated from the movement of the 50%-concentration point and corrected for deviations from the viscosity of water at 20°.

Gel electrophoresis. The method used was described by Richards, Coll & Gratzner (1965). Polyacrylamide gel (10%, w/v) was prepared by mixing 1 vol. of 50% (w/v) Cyanogum 41 (obtained from British Drug Houses Ltd., Poole, Dorset) in 0.486M-tris-0.1N-HCl buffer, pH8.47, with 0.1 vol. of dimethylaminoethyl cyanide and 0.1 vol. of aq. 10% (w/v) ammonium persulphate. Portions (2.5ml.) of the gel were transferred to glass tubes (9mm. diam.) sealed at one end with Parafilm, held vertically and left overnight to polymerize. RNA (0.25mg. in 0.25ml. of 5%, w/v, sucrose) was applied to the gel, layered with 0.0033M-tris-0.03M-barbituric acid buffer, pH7.0, which also served as the medium for electrophoresis (300V, 30mA), which was continued for about 1hr. The gels were removed, left overnight

in 1% (w/v) acridine orange–2% (w/v) lanthanum acetate in 15% (v/v) acetic acid. The unbound dye was later removed by electrophoresis in 10% (v/v) acetic acid.

Estimation of the fraction of oligonucleotides produced in hydrolysis. Samples of RNA were removed and each was added to an equal volume of 0.01 M-La(NO₃)₃ in 0.5 N-HClO₄ at 0°. The precipitate was removed by centrifuging for 10 min. at 3000g (MSE Mistral centrifuge).

Spectrophotometry. Absorption and difference spectra were measured with a Unicam SP.700 spectrophotometer. The sample-cell holder was an electrically heated copper block (Cox, 1963a). The observed extinctions measured at temperatures other than 20° were corrected for the thermal expansion of water.

Measurement of pH. An E.I.L. model 39A pH-meter (Electronic Instruments Ltd., Richmond, Surrey) was used in conjunction with electrodes covering the range pH 0–14.

RESULTS

Effect of ionic strength and temperature on the spectrum of oligonucleotides

OligoA. The difference in the spectrum over the range 220–320 mμ of a solution of oligoA maintained at 25° and an equivalent solution heated to temperatures in the range 25–95° was measured. The difference in the spectrum observed on heating to 87.5° in 0.1 M-potassium phosphate buffer, pH 7.0, is given in Fig. 3(a). A maximum was noticed at 260 mμ and the extinction decreased over the range 275–285 mμ. The form of the difference spectrum is similar to the denaturation spectrum of poly(A + U) shown as the broken line in Fig. 3(a). Similar difference spectra were obtained on heating to the other temperatures and the dependence of E_{260} and E_{280} on temperature is given in Fig. 4(a). Identical changes in the spectrum were observed when the solvent was 0.01 M-potassium phosphate buffer, pH 7, and the values of ΔE_{260} and ΔE_{280} obtained on raising the temperature coincided with those obtained (Fig. 4a) when the solvent was 0.1 M-potassium phosphate buffer. Increasing the buffer concentration to 1 M led to a small increase in ΔE_{260} obtained on heating to 50°. The same increment in E_{260} was also obtained when oligoA was heated in water in the absence of any added electrolyte as in 0.1 M solutions. The dependence of the spectrum of oligoA on temperature appears to be independent of the concentration of added electrolyte, at least up to 0.1 M-potassium phosphate buffer.

OligoG. The difference in the spectrum of oligoG observed on heating from 25° to 87.5° given in Fig. 3(b) is identical in form with the denaturation spectrum of polyG (Pochon & Michelson, 1965), with maxima at 245 and 275 mμ and a minimum at 260 mμ. Both E_{260} and E_{280} increased to the same extent within experimental error (see also Fig. 4b). A decrease in extinction over the range 295–305 mμ

Fig. 3. Denaturation spectra of oligonucleotides. In each case the change in the spectrum was measured on increasing the temperature from 25° to 87.5° and a correction was applied for changes in concentration due to the thermal expansion of water. The difference in the spectrum was essentially the same in water, 0.01 M-sodium phosphate buffer, pH 7.0, and 0.1 M-sodium phosphate buffer, pH 7.0. The difference spectra in 0.1 M-sodium phosphate buffer, pH 7.0, are given in the Figure. (a) —, Values observed for oligoA, $E_{1.04}$ at 25°; ○—○, denaturation spectrum of poly(A + U) (data of Fresco *et al.* 1963). (b) OligoG, E_{260} 0.845 at 25°. (c) OligoC, E_{260} 0.796 at 25°.

was observed. The changes in the spectrum brought about by heat did not depend on the concentration of potassium phosphate at least up to 0.1 M.

OligoC. The difference in the spectrum of oligoC (Fig. 3c) noticed on raising the temperature from

Fig. 4. Dependence of E_{260} and E_{280} of oligonucleotides in 0.1M-sodium phosphate buffer, pH 7.0, on temperature. (a) OligoA. (b) OligoG. (c) OligoC. The increments refer to solutions of E_{260} 1.0 at 25° in each case. $\circ$, ΔE_{260} ; $\bullet$, ΔE_{280} .

25° to 87.5° had maxima at 245 and 280m μ and minima at 230 and 260m μ . In contrast with the difference spectrum of both oligoA and oligoG the value of E in the region of 230m μ is substantial. The value of ΔE_{280} was about 2.7 times that of ΔE_{260} and an increase in E_{300} was observed. The values of ΔE_{260} and ΔE_{280} over the range 25–95° that are given in Fig. 4(c) were the same in both 0.01M- and 0.1M-potassium phosphate buffer.

Mixture of oligoA and oligoG. Equal volumes of solutions of oligoA (E_{260} 1.0 at 25°) and oligoG (E_{260} 1.0 at 25°) in 0.1M-potassium phosphate buffer were mixed and the changes in the spectrum on heating to 95° were measured. The increments in E_{260} and E_{280} observed were within 1% of the values calculated from Figs. 4(a) and 4(b). The observed difference spectra are given in Fig. 5(c). The difference spectrum of the mixture, for a particular temperature range, e.g. 45–55°, was calculated in the following way. First, the difference spectrum observed on heating oligoA to 87.5° (Fig. 3a) was reduced, first by (0.5/1.04) to correspond to the concentration of oligoA in the mixture and then by $(\Delta E_{260(87.5)} - \Delta E_{260(25)}) / (\Delta E_{260(85)} - \Delta E_{260(25)})$, by using the data of Fig. 4(a), to obtain the precise

Fig. 5. Comparison of the observed and calculated difference spectra of a mixture of equal volumes of oligoA (E_{260} 1.0 at 25°) and oligoG (E_{260} 1.0 at 25°) in 0.1M-sodium phosphate buffer, pH 7.0. (a) —, Calculated values of ΔE for the range 75–95° from the data of Figs. 4(a), 4(b), 5(a) and 5(b) as described in the text; $\bullet$, observed values (see c). (b) —, Values of ΔE calculated as in (a) for the range 45–55°; $\circ$, observed values (see c). (c) Difference spectra (E_{260} 1.0 at 25°; E_{280} 0.5 for both oligoA and oligoG) corrected for the thermal expansion of water observed on raising the temperature from 25° to 35°, 45°, 55°, 65°, 75°, 85° and 95° successively.

increments over the range $T_2 - T_1$; then the contribution of oligoG to the difference spectrum was calculated in the same way from the data in Figs. 3(b) and 4(b); finally, the two contributions were added. The calculated and observed difference spectra for the ranges 55–45° and 95–75° are given in Figs. 5(a) and 5(b) respectively. The agreement between the observed and calculated values indicates that the results are reproducible and the differences in the denaturation spectrum can be distinguished.

Hydrolysis of RNA by alkali

A solution of unfractionated RNA (approx. 2mg./ml.) was made 0.4N with respect to potassium hydroxide at 22°. Samples were then withdrawn

after 10, 15, 20, 25 and 30 min. and neutralized immediately by reaction with an equivalent solute, 0.4N-hydrochloric acid, made 0.2M with respect to sodium dihydrogen phosphate. The RNA was precipitated by the addition of 2 vol. of ethanol and redissolved in 0.1M-phosphate buffer, pH 7.

The sedimentation pattern of a dilute solution (0.05 g./l.) of each digest was studied at about 10°. The distribution of sedimentation coefficients is given in Fig. 6 for each digest. The sedimentation properties of RNA before hydrolysis were described by Cox & Arnstein (1963). The sedimentation coefficients ($S_{20,w}$) calculated from the movement of the 50%-concentration point were found to be 5.9, 3.6, 3.5, 3.0 and 2.8 s after hydrolysis for 10, 15, 20, 25 and 30 min. respectively. The number of scissions ($(S_{i=0}^2/S_i^2 - 1)$) was calculated by using an average value of 24 s for $S_{20,w}$ at zero time, and was found to be a linear function of the time spent at high pH (Fig. 7).

The behaviour of the fragments obtained after hydrolysis for 10, 15 and 30 min. on electrophoresis in polyacrylamide gels was also studied (see Fig. 8). In each case a broad ill-defined zone was observed compared with a sharp band found for unfractionated *E. coli* s-RNA. The R_f values of the bands were in agreement with the sedimentation properties, as reported by McPhie, Hounsell & Gratzer (1966).

The denaturation spectrum of unfractionated

RNA in 0.1M- and 0.01M-sodium phosphate buffer was described by Cox (1966a). In solutions of 0.1M-phosphate buffer, E_{260} increased from 1.0 at 25° by 0.165 to 1.165 on heating to 70°, whereas ΔE_{280} was 0.09. Increasing the temperature from 70° to 95° led to a further increase of 0.10 in E_{260} and of 0.10 in E_{280} . When the solvent was 0.01M-phosphate buffer denaturation took place at lower temperatures. E_{260} increased from 1.0 at 25° by 0.145 to 1.145 at 60°, and E_{280} increased by 0.085. When the temperature was increased from 60° to 95° both E_{260} and E_{280} increased by 0.10.

The RNA fragment obtained after only 10 min. hydrolysis was found to have very different denaturation properties. The melting range was broadened and $\Delta E_{280}/\Delta E_{260}$ was substantially increased compared with the control. In 0.1M-phosphate buffer, E_{260} increased from 1.0 at 25° by 0.135 to 1.135 on heating to 70°, but E_{280} increased by 0.10, and a further increase of 0.07 was obtained in both E_{260} and E_{280} on heating further to 95°. In 0.01M-phosphate buffer the total increment in E_{260} was 0.175, whereas E_{280} increased by 0.165.

Fig. 6. Sedimentation patterns of RNA after 10 min. (curve I), 20 min. (curve II) and 30 min. (curve III) in 0.4N-KOH at 25°.

Fig. 7. Number of scissions [calculated from $(S_{20,w})^2_{i=0}/(S_{20,w})^2_i - 1$] induced in RNA by hydrolysis (a) in 0.4N-KOH at 25°, (b) by ribonuclease. ○, Calculated from $S_{20,w}$ observed at 25°; ●, calculated from $S_{20,w}$ obtained by extrapolation to zero time of heating at 85° (see Fig. 12).

The fragments isolated after longer periods of hydrolysis were found to have essentially the same denaturation properties in 0.01 M-phosphate buffer as the fragments isolated after 10 min. However, as hydrolysis increased the effect of electrolyte concentration on the denaturation spectrum diminished, so that after 20 min. denaturation was almost the same in 0.01 M- as in 0.1 M-phosphate buffer. The denaturation studies are summarized in Fig. 9, where the changes in E_{260} and E_{280} are given as fractions of the extinction at 95°.

Enzymic hydrolysis of ribosomal RNA

Reticulocyte ribosomal RNA (4.9 mg.) in 0.1 M-potassium phosphate buffer, pH 7 (1.15 ml.), was treated with ribonuclease (0.15 μ g., i.e. 32.7 mg. of RNA/ μ g. of ribonuclease) at 22°. Samples (0.25 ml.) were withdrawn after 5, 10, 40 and 80 min. and each was immediately added to 0.1 ml. of suspension of bentonite (10 mg./ml.) in potassium phosphate buffer.

In another experiment the ratio of ribonuclease to RNA (19.6 mg.) in potassium phosphate buffer

(7.8 ml.) was treated with 7.5 μ g. of ribonuclease at 22°. At 15 min. intervals a sample (0.1 ml. in the early stages, then 0.05 ml.) was withdrawn and added to an equal volume of 0.01 M-lanthanum nitrate in 0.5 N-perchloric acid at 0°. The precipitate was separated by centrifuging and the spectrum of the supernatant, diluted with 0.1 M-potassium phosphate buffer, was measured and E_{260} recorded (see Fig. 11a). At the same time, another sample was added to an equal volume of a suspension of bentonite (10 mg./ml.) in potassium phosphate buffer. Preliminary experiments showed that this amount of bentonite was sufficient to stop further enzymic hydrolysis. Samples were withdrawn at 15 min. intervals for 3 hr. and a further sample was then removed after 20 hr. The sedimentation properties, denaturation spectra and in some cases the behaviour of the fragments on electrophoresis in 10% polyacrylamide gels were then examined. To compare the results of the two experiments, it is assumed that, if the amount of enzyme used in the first experiments was increased 12.5-fold to the ratio of RNA to ribonuclease in the second series, the same effects would be observed in 1/12.5 of the

Fig. 8. Electrophoresis of hydrolysis products of RNA in 10% polyacrylamide gels. (a), (b) and (c) refer to RNA hydrolysed by alkali according to the method of Spencer & Poole (1965) for 10, 15 and 30 min. respectively. The fragments were characterized by $S_{20,w}$ values (in 0.01 M-potassium phosphate buffer, pH 7.0) of respectively 5.9, 3.6 and 2.8 s. (d), (e), (f) and (g) respectively were obtained on electrophoresis after digestion with ribonuclease for 6.4, 30, 90 and 135 min.; the $S_{20,w}$ values were 7.3, 3.3, 3.2 and 3.2 s (before 'hidden' breaks were revealed by heating to 85°). The conditions for electrophoresis are given in the Experimental section. (h) Unfractionated s-RNA from *E. coli*.

time, namely 24sec., 48sec., 3.2min. and 6.4min. respectively (the values used below).

Sedimentation properties and the release of acid-soluble fragments. The sedimentation pattern of each sample (0.05g./l.) in 0.01M-sodium phosphate buffer, pH 7, was studied at about 10°. Bentonite (0.1mg./ml.) was present throughout to minimize

any further enzymic hydrolysis. The sedimenting boundary was heterogeneous (Fig. 10) after only 0.4min. digestion with ribonuclease. An immediate rapid decrease in $S_{20,w}$ to 3.3s was found during the first 30min. of hydrolysis. The size of the sedimenting species then decreased very slowly to 2.5s after 1200min. (Fig. 11). However, the sedimenting

Fig. 9. Dependence of E_{280} (a and c) and E_{280} (b and d) on temperature after hydrolysis by alkali. (a) and (b) Solvent 0.1M-sodium phosphate buffer, pH 7.0. (c) and (d) Solvent 0.01M-sodium phosphate buffer, pH 7.0. Curves I, II, III, IV, V and VI refer respectively to RNA kept at 0.4N-KOH for 0, 10, 15, 20, 25 and 30 min.

Fig. 10. Sedimentation patterns of RNA after hydrolysis with ribonuclease for 0.4 min. (a), 0.8 min. (b), 3.2 min. (c) and 6.4 min. (d). Measurements were made at about 10° in 0.01 M-sodium phosphate buffer, pH 7.0. Values obtained before (○) and after (●) heating at 85° for 3 min. are shown.

species formed a smaller fraction of the total material as hydrolysis progressed. The fraction of the extinction at 260 mμ that is not precipitated by lanthanum nitrate increased as $S_{20,w}$ decreased. The acid-soluble fraction increased from 0 to 6.4% during the first 30 min. and then regularly to 14.4% after 180 min. and to 30.4% after 1200 min. (Fig. 11a).

The heterogeneity of the digests shown by the broad distribution of sedimentation coefficients was vividly demonstrated by electrophoresis in 10% polyacrylamide gels (Fig. 8). In contrast with the diffuse zones obtained with RNA hydrolysed by alkali, enzymic hydrolysis yielded digests that separated into at least ten distinct bands on electrophoresis. The distribution of the zones corresponded to the spread of S values. The major changes were complete after 30 min. hydrolysis. Many of the same bands were still present after hydrolysis for 180 min., although the diffuse zone close to the solvent front was more pronounced and the faster-travelling zones themselves were also more diffuse.

The sedimentation properties of the digests were also studied after secondary structure was destroyed

by heating to 85° in 0.01 M-sodium phosphate buffer, pH 7. Bentonite (0.1 mg./ml.) was present throughout. A dilute solution (0.5 g. of RNA/l.) was heated to 85° and samples were removed after 3, 12 and 30 min., cooled rapidly, stored at -12° and subsequently centrifuged at about 10°. Heating for 3 min. at 85° led to a marked decrease in S value (Fig. 10) and possibly also a decrease in heterogeneity (see also Hall & Doty, 1958). Further heating did not change the sedimentation pattern appreciably. For each digest $1/(S_{20,w})^2$ was plotted against the time spent at 85°. A straight line was drawn through the three points since the observed variation in $S_{20,w}$ after 3 min. is within the range expected for thermal hydrolysis (see Möller & Boedtker, 1961). Extrapolation to zero time is considered to yield the value of $1/(S_{20,w})^2$ that would be observed if secondary structure were destroyed at about 22°. The extrapolated value was considerably higher than that of the unheated solution. This difference is attributed to secondary structure 'hiding' chain scissions. The results for the digests obtained after 0.4, 0.8, 3.2 and 6.4 min. are given in Fig. 12. The sedimentation pattern of the fragments obtained after 30 min. hydrolysis was 3.3 s before and 2.91 s after heating.

The initial rapid decrease in the molecular weight of RNA during the first 10 min. digestion with ribonuclease compares with the course of hydrolysis by alkali. This comparison is emphasized in the plot of $(S_{20,w}^2/S_0^2 - 1)$ against time (Fig. 7). Values of S_0^2 were calculated from the sedimentation coefficients of the digests before heating to 85° and from the sedimentation coefficient obtained by extrapolation to zero time at 85°. The plots show that in the early stage of enzymic hydrolysis a very high proportion of scissions are 'hidden' by secondary structure. This fraction decreased as hydrolysis progressed (Fig. 11). The fraction breaks 'hidden' before heating was calculated from eqn. (32) to be 0.8 after 0.4 min., 0.7 after 0.8 min., 0.6 after 3.2 min., 0.7 after 6.4 min. and 0.23 after 30 min. hydrolysis.

The number of chain scissions calculated from the properties of the sedimenting species is to be compared with the probability, $\bar{p}$, of chain scission calculated from eqn. (31) by using the values of $S_{20,w}$ obtained by extrapolation to zero time of heating at 85° (see Fig. 11b). After 30 min. the contribution of the acid-soluble fraction to $\bar{p}$ becomes dominant so that the plot of $\bar{p}$ against time has the same form as the plot of the percentage of acid-soluble fragments against time.

Denaturation studies. The principal changes in secondary structure due to enzymic hydrolysis were completed after 30 min. and were followed by comparing the changes in extinction obtained over various ranges of temperature on denaturation of RNA in 0.01 M- and 0.1 M-potassium phosphate

Fig. 11. (a) Dependence of $S_{20,w}$ before (○) and after (●, extrapolated values) heating at 85° and the release of acid-soluble fragments (Δ) on the period of digestion with ribonuclease. (b) Probability, $\bar{p}$, of a phosphodiester bond being hydrolysed [calculated from the data of (a) by using eqn. (11)] as a function of the time of digestion with ribonuclease.

Fig. 12. Effect of incubation at 85° on the molecular weight of RNA after digestion with ribonuclease. The ordinate $100/(S_{20,w})^2$ is proportional to the reciprocal of the molecular weight. ○, After 0.2 min. digestion; ●, after 0.4 min. digestion; Δ, after 3.2 min. digestion; □, after 6.4 min. digestion. The conditions for centrifuging were the same as in Fig. 8, and bentonite (1.0 mg./ml.) was present throughout.

buffer, pH 7.0. The difference in the spectrum of a solution maintained at about 25° and an equivalent solution heated to temperatures in the range 25–95° was measured over the range 220–320 mμ. To compare the denaturation spectra of different solutions, the values of E_{260} and E_{280} observed at 95° (which more closely approach the extinction of an equivalent mixture of nucleotides) were taken as points of reference. The dependence of E_{260} and E_{280} on temperature for representative digests in 0.01 M- and 0.1 M-sodium phosphate buffer are given in Fig. 13. The same trends were observed as on hydrolysis with alkali (compare Figs. 9 and 13); the temperature range over which 'melting' occurred became broader and less sensitive to electrolyte concentration, and the absolute increments in E_{260} and E_{280} tended to become equal. Even after 20 hr. digestion with ribonuclease the spectrum was still dependent on temperature. The changes in the spectrum with temperature, over the range 220–

Fig. 13. Comparison of the increments in E_{260} (a and c) and E_{280} (b and d) on digestion of RNA with ribonuclease for: ■, 0 min.; □, 6 min.; ○, 30 min.; ●, 1200 min. (a and b) or 180 min. (c and d). The solvent was 0.1 M- (a and b) or 0.01 M- (c and d) potassium phosphate buffer, pH 7.0.

300 m μ , were reversible to within about 2% of the original E_{260} value at 25° and were essentially reproduced on heating for a second time.

Unfractionated reticulocyte ribosomal RNA is characterized by the appearance of two principal difference spectra at different temperature ranges (Cox, 1966a). In 0.1 M-sodium phosphate buffer E_{260}

and E_{280} increased mainly above 50°, between 50° and 70° the difference spectrum ($E_{(T)} - E_{(50)}$) had a single maximum at about 260 m μ and $\Delta E_{280}/\Delta E_{260}$ was about 0.6, whereas above 70° the difference spectrum ($E_{(T)} - E_{(70)}$) had maxima at about 250 and 274 m μ and a minimum at 260 m μ , and the $\Delta E_{280}/\Delta E_{260}$ ratio was about 1.0. In 0.01 M-sodium

Fig. 14. Dependence of $E(T_2) - E(T_1)$ (where $T_2 > T_1$) on the time of digestion with ribonuclease. ○, E_{260} ; ●, E_{280} . (a) shows the formation and subsequent destruction of secondary structure as digestion proceeds. Measurements were made in 0.1M-potassium phosphate buffer, pH 7.0. (b) and (c) illustrate the change in the original secondary structure characterized by $E_{280}/E_{260} = 0.6$ on digestion. Measurements were made in 0.1M- (b) and 0.01M- (c) potassium phosphate buffer, pH 7.0.

phosphate buffer E increased over a lower temperature range and the bimodal difference spectrum was observed above about 60°.

Small but significant changes in the dependence of E on temperature were noticed after 24 sec. hydrolysis, notably an increase in both ΔE_{260} and ΔE_{280} over the range 25–50° (Figs. 13 and 14), although the total increment over the range 25–95° was slightly decreased (Figs. 13 and 14). This trend continued on hydrolysis for up to 3 min. Further hydrolysis led to a decrease in ΔE_{260} and ΔE_{280} over the range 25–50° as well as at higher temperatures (Fig. 13). The increment in ΔE_{260} and ΔE_{280} observed over the range 50–70° (solvent 0.1M-potassium phosphate buffer) and 30–60° (solvent 0.01M-potassium phosphate buffer) decreased rapidly in the early stages (less than 15 min.) of the digestion with ribonuclease and then reached plateau values that were hardly affected by further hydrolysis; the values of ΔE_{260} and ΔE_{280} approached one another after hydrolysis for about

Fig. 15. Dependence of $E(T_2) - E(T_1)$ on the time of digestion with ribonuclease. In both (a) and (b) $\Delta E_{280}/\Delta E_{260} = 1.0$ before digestion. The increments refer to a solution of $E_{260} 1.0$ at 25°. ○, E_{260} ; ●, E_{280} . Measurements were made in 0.1M- (a) and 0.01M- (b) potassium phosphate buffer, pH 7.0.

Fig. 16. Difference spectra observed on heating RNA in 0.1M-potassium phosphate buffer, pH 7.0, after digestion with ribonuclease for 30 min. The differences in E were observed on increasing the temperature from 25° to 33.5°, 43.5°, 53.5°, 62°, 71°, 80° and 95° successively. At 25°, $E_{260} = 0.875$. A correction was applied for the thermal expansion of water. In each case the increments refer to a solution where $E_{260} = 1.0$ at 25°.

30 min., i.e. $\Delta E_{280}/\Delta E_{260}$ increased from about 0.6 before hydrolysis to about 1.0 after 30 min.

The values of ΔE_{260} and ΔE_{280} over the range 75–95° in 0.1M-potassium phosphate buffer and

65–95° in 0.01M-potassium phosphate buffer also decreased on hydrolysis to a plateau value after hydrolysis for 30min., although the $\Delta E_{280}/\Delta E_{260}$ ratio was not affected and remained about 0.8–1.0 throughout (Figs. 13 and 15).

The difference spectra found on heating RNA after 30min. digestion with enzyme are given in Fig. 16. Above about 50° the difference spectrum ($E_{(T)} - E_{(50)}$) was bimodal, $\Delta E_{280}/\Delta E_{260}$ was close to unity, and the melting range was hardly affected by increasing the concentration of phosphate buffer from 0.01M to 0.1M. Further hydrolysis lowered the temperature above which the bimodal difference spectrum was first observed. When the pH was adjusted to 3.8–4.0 the effect on the difference spectrum contrasted with that found for the intact molecule since $\Delta E_{280}/\Delta E_{260}$ decreased and the

increments in E_{260} and E_{280} were reduced by about 20% of the increments found at pH 7.

A direct comparison of the effects of alkaline and enzymic hydrolysis on the denaturation spectrum was attempted. The increments in E_{260} and E_{280} found after 10, 15 and 25min. hydrolysis with alkali are given as the full line in Fig. 17. The increments noted for fragments of about the same size (after 3.2, 6.4 and 30min. hydrolysis), as measured by $S_{20,w}$ extrapolated to zero time of heating at 85° (see Fig. 8), are shown on the corresponding diagrams as the circles. It is clear that for a given size of fragment there is a close correspondence in the denaturation spectrum irrespective of the conditions of hydrolysis. The increments in E_{260} and E_{280} observed after hydrolysis for 20hr. are given for comparison.

Fig. 17. Comparison of the denaturation profile of fragments of RNA produced by hydrolysis with alkali or with ribonuclease. —, Data for alkaline hydrolysates. O, ΔE_{260} , and ●, ΔE_{280} , noted for enzymic hydrolysates: (a) after 10 min. in 0.4N-KOH compared with 3.2 min. digestion with enzyme; (b) after 15 min. in 0.4N-KOH compared with 6.4 min. digestion with enzyme; (c) after 25 min. in 0.4N-KOH compared with 30 min. digestion with enzyme; (d) after 20hr. digestion with enzyme. Values of $S_{20,w}$ were about the same for each pair. The solvent was 0.1M-sodium or -potassium phosphate buffer, pH 7.0. In each case at 25° $E_{260} = 1.0$.

DISCUSSION

Effect of ionic strength and temperature on the spectrum of model compounds

There is strong evidence that the tendency of adjacent base pairs along a polynucleotide chain to stack one upon another gives rise to the secondary structure of polyA (Leng & Felsenfeld, 1966; Brahms *et al.* 1966) and polyC (Fasman *et al.* 1964) at pH 7. OligoG probably also forms secondary structure in the same way (see Cox, 1966b). It is

Fig. 18. Comparison of the 'denaturation' spectrum of the mixture of oligoA and oligoG (see Figs. 4 and 5) with the denaturation spectrum for A-U and G-C base pairs. (a) —, calculated for 40% of G-C and 60% of A-U base pairs, according to Fresco *et al.* (1963); ----, observed values for the range 25–87.5°. (b) Analysis of the observed data for oligoA and oligoG by using the data for A-U and G-C base pairs in eqn. (34). (c) Analysis of the observed difference spectrum by using the data for oligoA and oligoG in eqn. (36).

Fig. 19. Effects of enzymic hydrolysis on $\Delta E_t / \Delta E_0$ calculated for different types of secondary structure. ΔE_t is the increment observed on heating from 25° to 95° for the first time. The suffix shows the time of digestion with enzyme. Curve I, ΔE_0 due entirely to double-helical segments that are not attacked by ribonuclease; curves II, ΔE_0 due entirely to stacking (i.e. $2N+b=2$ in eqn. 27); curves III, combination of curves I and II; curves IV, ΔE_0 due to double-helical segments that are attacked by ribonuclease at the same rate as single-stranded regions ($2N+b=10$ in eqn. 27); stacking is not important at any temperature; curves V, as for curves IV, but the double-helical segment is now assumed to revert to a single-stranded stacked form on heating ($2N+b=10$, $\Delta E_0^s / \Delta E_0 = 0.4$); curves VI, as for curves IV, but with $2N+b=35$. O, Digestion with ribonuclease; ●, digestion with 0.4N-KOH. Measurements were made in 0.1M- (b) or 0.01M- (c) potassium phosphate buffer, pH 7.0.

evident from Figs. 4(a) and 19 that the difference in the spectrum of oligoA observed on heating is very similar to the denaturation spectrum reported for A-U base pairs (Fresco *et al.* 1963; Cox, 1966b), and from Figs. 4(b) and 4(c) that combinations of the difference spectra observed on heating oligoG and oligoC reproduce various denaturation spectra reported for G-C base pairs (Fresco *et al.* 1963; Pochon & Michelson, 1965; Cox, 1966b). Further, the difference spectrum (Figs. 5c and 18a) observed on heating a mixture of oligoA and oligoG from 25° to 87.5° is similar in form to the denaturation spectrum of a mixture of 54% of A-U base pairs and 46% of G-C base pairs calculated from the data of Fresco *et al.* (1963). These authors assume that:

$$\frac{\Delta E_{\lambda}}{C_m} = f_{G-C} \Delta \epsilon_{G-C} + f_{A-U} \Delta \epsilon_{A-U} \quad (33)$$

where $\Delta \epsilon_{A-U}$ and $\Delta \epsilon_{G-C}$ are the increments in the extinction coefficient (per g. atom of P) at $\lambda m\mu$ on denaturation of A-U and G-C base pairs respectively, and C_m is the molar concentration (g. atoms of P/l.) of nucleotides in the solution studied.

Dividing by $\Delta \epsilon_{A-U}$ and multiplying throughout by C leads to:

$$\frac{\Delta E_{\lambda}}{\Delta \epsilon_{A-U}(\lambda)} = C_m f_{G-C} \cdot \frac{\Delta \epsilon_{G-C}}{\Delta \epsilon_{A-U}} + C_m f_{A-U} \quad (34)$$

Thus a plot of $\Delta E_{\lambda}/\Delta \epsilon_{A-U}$ against $\Delta \epsilon_{G-C}/\Delta \epsilon_{A-U}$ should be linear, the ratio of the slope to the intercept on the ordinate yields f_{A-U}/f_{G-C} . When the values of ΔE_{λ} observed for the mixture of oligoA and oligoG were used in conjunction with $\Delta \epsilon_{A-U}$ and $\Delta \epsilon_{G-C}$ a straight line was observed (Fig. 19b), and f_{A-U} and f_{G-C} were calculated to be 56.2 and 43.8% respectively.

Although the difference spectra observed for oligoA and oligoG were not placed on a molar basis, eqn. (33) may be written as:

$$\frac{\Delta E_{\lambda}}{C} = f_G \cdot \frac{\Delta E_{G(\lambda)}}{C_G^0} + f_A \cdot \frac{\Delta E_{A(\lambda)}}{C_A^0} \quad (35)$$

where ΔE_G is the increment at $\lambda m\mu$ observed on heating a solution of oligoG of concentration C_G^0 to 95°. The form analogous to eqn. (34) is:

$$\frac{\Delta E_{\lambda}}{\Delta E_{A(\lambda)}} = \frac{C}{C_G^0} \cdot f_G \cdot \frac{\Delta E_{G(\lambda)}}{\Delta E_{A(\lambda)}} + \frac{C}{C_A^0} \cdot f_A \quad (36)$$

and a plot of $\Delta E_{\lambda}/\Delta E_{A(\lambda)}$ against $\Delta E_{G(\lambda)}/\Delta E_{A(\lambda)}$ should lead to a straight line where the ratio of slope to intercept is $(f_G/f_A) \cdot (C_G^0/C_A^0)$. If the unit of concentration is taken to be E_{260} 1.0 at 25° then $f_G/f_A = 1.0$, in accord with the known composition of the solution (Fig. 18c).

Evidently, for a polynucleotide such as RNA, where both base pairing and base stacking are possible, it is difficult to distinguish between these two effects on the basis of the denaturation spectrum obtained at one ionic strength at pH 7.

However, it appears from the results presented above that the changes in the extent of 'stacking' over a particular temperature range, measured by changes in the spectrum, are not affected by increasing the concentration of electrolyte (at least up to 0.1 M-potassium phosphate). These observations are in accord with the work of Barszcz & Shugar (1964) and Vournakis, Scheraga, Rushizky & Sober (1966), who found that ApApCp has appreciable structure in the absence of added electrolyte that is comparable with that of ApAp in 0.15 M-sodium chloride-0.1 M-sodium phosphate buffer, pH 6.88 (Poland, Vournakis & Scheraga, 1966). In contrast, secondary structure due to base pairing is sensitive to the electrolyte concentration (Dove & Davidson, 1962).

Hydrolysis of RNA

Chain scission. The hydrolysis of RNA by alkali at about pH 13 appears to take place at random, judged by the broad distribution of the sedimenting species (Fig. 10), by the diffuse zones observed on gel electrophoresis (Fig. 8) and by the observation that the number of scissions appears to be directly proportional to the period of hydrolysis (cf. Möller & Boedtker, 1961).

In contrast, the enzymic hydrolysis of RNA led to the appearance of many distinct species (cf. Aronson & McCarthy, 1961; Huppert & Pelmont, 1962), as shown by gel electrophoresis (McPhie *et al.* 1966; Gould, 1966). This result is in accord with the simple hairpin-loop model. The immediate decrease in molecular weight (see Figs. 7 and 11) is attributed to chain scission within the flexible segments joining the hairpin loops (Fig. 1a), and possibly also to scissions within the double-helical segment but within N^0 residues of the open end, AE, of the loop (Fig. 1a). The molecular weight would be expected to change by multiples of $(2N + b)$. It has been reported (Gould, 1966) that the zones seen in polyacrylamide gels correspond to species differing in molecular weight by 10000. This increment is taken as a tentative measure of $(2N + b)$, which, on this basis, corresponds to about 30 residues. The presence of double-helical secondary structure is sufficient to account for the formation of fragments (before 'hidden' breaks are revealed) differing by multiples of $(2N + b)$ residues; it is not necessary to make any assumption about the relative susceptibility to hydrolysis of linkages in double-helical and single-stranded segments of RNA. The molecular-weight distribution is very broad, as shown by the

sedimentation profile and gel electrophoresis, but no quantitative analysis (see eqns. 9 and 10) was made.

The observation that initially 70–80% of the chain scissions have no immediate effect on molecular weight is also consistent with the hairpin-loop model. The calculation of a precise value for the fraction of hidden breaks depends on a knowledge of the relation between M_w and M_n before and after 'hidden' breaks are revealed. We have assumed that the relation is the same in both cases. The interpretation of the fraction of 'hidden' breaks also depends on the rate at which double-helical segments are attacked. If single-stranded segments only are susceptible to hydrolysis by ribonuclease, then the ratio of 'hidden' breaks to breaks that are immediately observed reflects the relative lengths of the single-stranded section of the hairpin loop (BCD in Fig. 1a) and the flexible segment joining two hairpin loops (EF in Fig. 1a), if unusually sensitive linkages and special sequences are not important ($\bar{p}_{BCD}/b = \bar{p}_{EF}/c$) (see eqn. 25). If the enzyme does not discriminate between double-helical and single-stranded structures, then the ratio of 'hidden' to apparent breaks reflects the relative lengths of the whole hairpin loop and the flexible segment joining one loop to another. Hence, provided that special sequences are not important, the hairpin loop ABCD is likely to be at least two to three times the length of the section EF (Fig. 1).

The fraction of scissions hidden by secondary structure decreased as the hydrolysis progressed (Fig. 7), in qualitative agreement with eqn. (23).

Changes in secondary structure. To provide a basis for interpreting the effects of hydrolysis on denaturation the dependence of $\Delta E_i/\Delta E_0$ on $\bar{p}$ is given in Fig. 19(a) for various hypotheses; ΔE_0 is the increment in E obtained on heating the intact ribosomal RNA over a particular temperature range, and ΔE_i is the increment observed over the same temperature range after hydrolysis for t_{min} . If the increment ΔE is due solely to denaturation of the $2N$ residues of the double-helical segments (i.e. stacking is negligible at all temperatures) and if double-helical segments are not attacked by ribonuclease, then ΔE_i will always be equal to ΔE_0 and $\bar{p}$ will reach a limiting value (curve I in Fig. 19a) of $\bar{p}(1-H)$ if the proportion of pyrimidine to purine residues is the same in double-helical and single-stranded regions of the chain. If the denaturation spectrum arises solely from 'stacking' then the ΔE_i will slowly decrease (curve II in Fig. 19a) as $\bar{p}$ increases, according to eqn. (27) where $2N+b=2$. If both types of secondary structure are present at 25° then curve III, intermediate between curves I and II, will be followed (Fig. 19a), depending on the proportion of residues in the two forms.

It is possible that the internucleotide linkages

within the double-helical segment are attacked by ribonuclease. When ribonuclease is not inhibited by secondary structure ($\bar{p}_{ab}/2N = \bar{p}_{BCD}/b = \bar{p}_{EF}/c$), ΔE_i will decrease according to eqn. (27) if stacking is not important. The smallest hairpin loop is unlikely to be less than ten residues ($2N+b=10$) and $\Delta E/\Delta E_0$ will have the form of curve IV in Fig. 19(a).

If the destruction of the double-helical segment leads to a single-stranded 'stacked' structure, then $\Delta E/\Delta E_0$ will follow a curve such as V in Fig. 19(a). If ΔE_0^* is the increment that would be observed at zero time if the segment forming a hairpin loop were in a single-stranded stacked form, and if f_{ab} and f_s respectively are the fractions calculated from eqn. (27) for $2N+b \geq 10$, and $2N+b=2$, then:

$$\Delta E/\Delta E_0 = f_{ab} + (1-f_{ab})(\Delta E_0^*/\Delta E_0)f_s \quad (37)$$

Curve V in Fig. 19(a) was calculated for values of $2N+b=10$ and $\Delta E_0^*/\Delta E_0=0.4$.

The results show that enzymic hydrolysis has an immediate effect on secondary structure (Figs. 13, 14 and 15), as measured by changes in the spectrum of RNA. Both the secondary structure 'melting' over the range 50–70° (solvent 0.1M-potassium phosphate buffer, pH 7) and that 'melting' over the range 65–95° (solvent 0.01M-potassium phosphate buffer, pH 7) were affected. These changes in the spectrum are given in Figs. 19(b) and 19(c) respectively as a function of $\bar{p}$.

Of the hypothetical models discussed above, curve V best fits our data. The agreement between values of $\Delta E_i/\Delta E_0$ for a given value of $\bar{p}$ obtained by hydrolysis by alkali and by enzyme is a strong indication that the enzyme does not discriminate between double-helical and amorphous segments of ribosomal RNA in 0.1M-phosphate buffer at 25°. This observation is surprising since the apparent resistance of double-stranded RNA to ribonuclease (judged by zone centrifuging) has been used (e.g. Montagnier & Sanders, 1963) to study the replicating forms of viral RNA. However, reo-virus RNA, which is known to be DNA-like, is attacked very slowly by ribonuclease at the salt concentration used in these studies (Shapiro, Bellamy, August & Joklik, 1966), and the rate of hydrolysis is increased when the ionic strength is lowered, even though hydrolysis took place at 30° below the temperature of thermal denaturation. It is not the double-helical structure itself that inhibits nuclease action. It cannot be assumed that a short double-helical segment of low thermal stability has the same resistance to ribonuclease as the high-molecular-weight double-helical structure. The observed decrease in molecular weight (Fig. 7), the immediate effect of enzymic hydrolysis on the denaturation spectrum (Figs. 9 and 19) and the observation that fragments of about the same $S_{20,w}$ have similar denaturation spectra irrespective

of whether hydrolysis was brought about by ribonuclease or by alkali (Figs. 17 and 19) all suggest that the enzyme attacks ribosomal RNA at random under our experimental conditions, i.e. $\bar{p} = p' = p'' = p'''$.

Stacking makes an appreciable contribution to the secondary structure persisting after hydrolysis for 30 min., judged by the breadth of the transition, by the reduced sensitivity of the 'melting' range towards ionic strength (Fig. 13) and by the denaturation spectrum noted at pH 3.8–4.0 when $\Delta E_{280}/\Delta E_{260}$ was less than the ratio noted at pH 7, showing that most of the cytosine residues do not undergo a co-operative transition on heating (cf. Cox, 1966a).

Stanley & Bock (1965) have shown that many di- and tri-nucleotides have appreciable hypochromism so that an appreciable contribution by single-stranded segments to the denaturation spectrum is to be expected. It was estimated from the data of Stanley & Bock (1965), with a knowledge of the nucleotide composition, that if reticulocyte RNA were hydrolysed to di- and tri-nucleotides E_{280} would increase by about 8% on heating from 25° to 95°, i.e. close to the increment observed after hydrolysis for 20 hr. (Figs. 13 and 17). PolyAU, which is a good model for RNA, provides a clear example of a polynucleotide that reverts to a single-stranded stacked form when double-helical secondary structure is destroyed, since it was shown by Cox (1966b) that the secondary structure of polyAU reverts to a form comparable with the alkaline form of polyA (cf. Cox 1963a), a form that is now known to be a single-stranded stacked structure.

It appears from Fig. 19(b) that the experimental results are best fitted by values of $2N + b = 15$ and $\Delta E_0^*/\Delta E_0 = 0.4$. All of the points lie within curves V and VIII, i.e. within the range 10–35 residues. This range would still apply even if $\bar{p}$ is grossly in error. The value of p calculated for the fragments obtained by alkaline hydrolysis are based on the assumption that $M_n = 0.5M_w$, a relation that we have no reason to doubt since degradation appears to take place at random, and on the relation between S and M_w given in eqn. (29). It is possible, but unlikely, that the exponent of 0.49 is in error. If the exponent were less, for example the relation:

$$S_{20,w} = kM^{0.43} \quad (38)$$

was found for polyA at pH 7 (Fresco & Doty, 1957), which is a single-stranded stacked structure, then the calculated value of $\bar{p}$ would be less than the true value so that the range calculated for $(2N + b)$ would be an overestimate. However, if the true exponent were 0.6 then the value $\bar{p}$ calculated from eqn. (29) would be greater than the true value so that we would underestimate $(2N + b)$. The

numbers of scissions calculated for a change in sedimentation coefficient from 24s to 3s are 126, 64 and 32 respectively for exponents of 0.43, 0.50 and 0.60. Even if the values of $\bar{p}$ calculated from eqn. (29) were twice the true value $(2N + b)$ would still be 35 or less. It is far more likely that the fragments more closely resemble a single-stranded stacked structure than a more tightly coiled structure, so that we may underestimate $\bar{p}$ and so overestimate $(2N + b)$. $\Delta E_i/\Delta E_0$ values over the range 50–70° are accurate to within ± 0.05 . Thus the upper limit for $(2N + b)$ is 35.

The values of p calculated for the fragments obtained by enzymic hydrolysis are subject to another error. It can no longer be assumed that $M_n = 0.5M_w$, although the evidence from these studies is that this relation does apply, since it appears that $\bar{p} = p' = p'' = p'''$, as discussed above, and since the distribution of sensitive linkages is likely to be close to random (Staehelin, 1961). However, if degradation is not random (which might be the case in the late stages of enzymic hydrolysis) M_n will be a smaller fraction of M_w , so that we may underestimate $\bar{p}$ and overestimate $(2N + b)$. The term 0.54 in eqn. (29) is an attempt to compensate for this effect. However, the term 0.54 is negligible when $\bar{p}$ is less than 0.5, but increases and indeed dominates after extensive hydrolysis. The values of $\bar{p} \geq 0.1$ may well be in error, but precision within this range is not critical.

We have taken a value of $\Delta E_0^*/\Delta E_0 = 0.4$, although a range from 0.3 to 0.5 would still be consistent with our data. If ΔE_0^* were negligible then $(2N + b)$ could not be greater than 10. We would underestimate $(2N + b)$ if $\Delta E_0^*/\Delta E_0$ is much greater than 0.4.

The values of $\Delta E/\Delta E_0$ for the range 65–95° (solvent 0.01M-potassium phosphate buffer) are accurate to ± 0.1 because ΔE_0 is 0.055, which is about half ΔE_0 for the range 50–70°. The scatter of points is consistent with values of $(2N + b)$ in the range 10–20 residues.

The results of this work provide an estimate of $(2N + b)$ that does not conflict with the values of N obtained by an analysis of the denaturation spectrum. The results also show that the secondary structure 'melting' over the range 25–95° is dependent on chain length, as would be expected for double-helical segments.

Finally, we infer that, to analyse the denaturation spectrum of RNA, it is essential to take into account the observation that the denaturation of RNA results in a transition from a partly double-helical form to a single-stranded stacked structure (cf. Fasman, Lindblow & Seaman, 1965). This is very important, since the precise form of the denaturation spectrum, the apparent breadth of the transition and the proportion of residues

Scheme 1. Suffixes dh, s, and am indicate the double-helical, single-stranded stacked and amorphous forms respectively. The equilibria enclosed within the broken line may be evaluated by studying the small fragments obtained by alkaline hydrolysis.

apparently 'melting' over a particular temperature range will depend on the contribution of the single-stranded stacked structure to the denaturation spectrum, and this may be different at various wavelengths. Thus the equilibria shown in Scheme 1 better describe the properties of RNA.

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