Physical Studies on Mixed Ribonucleic Acids from Yeast*

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ABSTRACT: Unfractionated yeast ribonucleic acid (RNA) was partially degraded with various concentrations of pancreatic ribonuclease at 25° in the presence of 0.15 M NaCl–0.005 M $MgCl_2$. Gel filtration of the digests on Sephadex G-75 in the same solvent yielded a high molecular weight product whose molecular parameters closely resembled those of intact RNA. If more than 35–40% digestion occurred, these RNA-like characteristics were rapidly lost. This RNA-like product was readily distinguishable from intact RNA by chromatography on Sephadex G-75 under conditions where secondary structure no longer existed (*i.e.*, buffered 7 M urea at 65°). After removal of urea, this component exhibited a much reduced sedimentation coefficient (2.25 S) whereas, after similar treatment, intact RNA remained virtually unchanged (4.0–4.1 S). The presence of Mg^{2+} appeared to be essential for the maintenance of this RNA-like structure since EDTA treatment of a digest prior to Sephadex fractionation yielded an elution profile in which the RNA-like material had given way to two new components of 3.55 and 1.35 S. These results seem to indicate that RNA consists of a double-stranded molecule containing several single-stranded regions, the removal of which does not greatly alter the size and shape of the molecule. It is felt that the "clover-leaf" model of RNA structure (Holley, R. W., Apgar, J., Everett, G. A., Madison, J. T., Marquis, M., Merrill, S. H., Penswick, R. J., and Zamer, A., *Science 147*, 1462 (1965)) best fulfills these requirements.

The central role played by RNA in protein biosynthesis has been well established and much is now known about the physical and biochemical properties of this RNA species (see Brown (1963) for a review). However, much less information has been forthcoming as to the secondary, and more especially the tertiary, structure of these molecules.

Physical studies (Tissières, 1959; Osawa, 1960; Luborsky and Cantoni, 1962) have shown that mixed RNAs exhibit little sedimentation or diffusion heterogeneity, indicating that RNA preparations, which are heterogeneous with respect to function, are probably fairly homogeneous with respect to gross structure and molecular weight. It has been shown by many workers (Fresco *et al.* (1960) and others) that RNAs have an ordered secondary structure consisting of helical regions formed by intramolecular hydrogen bonding. Felsenfeld and Sandeen (1962) showed that the hyperchromicity resulting from the thermal denaturation of RNAs could be interpreted as the sum of separate contributions from groups of A–U and G–C base pairs. On this basis a possible RNA structure, consisting of an A–U-rich helical region separated from a G–C-rich helical region by a single-stranded region was postulated (Figure 1A). This model seemed to gain support from the finding that several apparently pure single RNAs exhibit a biphasic thermal denaturation profile (Fresco, 1963).

McCully and Cantoni (1962), on the basis of sequence data obtained from partial RNase digests of RNA, proposed another model in which the polynucleotide chain folded back on itself to form a single looped structure in which only the terminal sequence (pCpCpA)[1] and a central region, containing the unusual bases and the coding triplet, did not participate in hydrogen-bond formation (Figure 1B).

This present work was undertaken in the belief that these possible RNA structures could be distinguished on the basis of their sensitivity to single-strand breaks. A single phosphodiester bond scission in the central region of the structure in Figure 1A would cause a dramatic reduction in molecular weight, whereas the same break would have very little effect on the structure in Figure 1B, assuming that conditions compatible with the maintenance of hydrogen bonds pertain.

In 1965, Holley *et al.* established the complete base sequence of alanyl-tRNA and demonstrated that Watson–Crick base pairing could give rise to structures of the type shown in Figure 1A,B and also to the clover-leaf model shown in Figure 1C.

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[1] Abbreviations used: pCpCpA, 5′-phosphocytidylyl-(3′–5′)-cytidylyl-(3′–5′)-adenosine; T_m, the temperature at which 50% of the total hyperchromicity owing to helix–coil transition has occurred. One optical density unit is defined as that amount of material per milliliter which in a 1-cm light path at 260 mμ gives a spectrophotometer reading of 1.

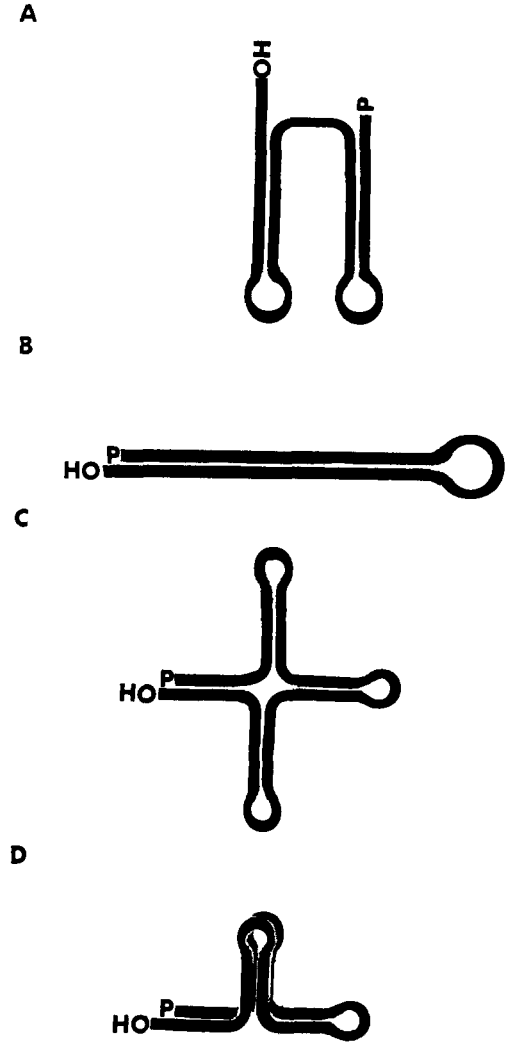

FIGURE 1: Diagrammatic representations of various RNA structural models. Parallel lines represent helical regions.

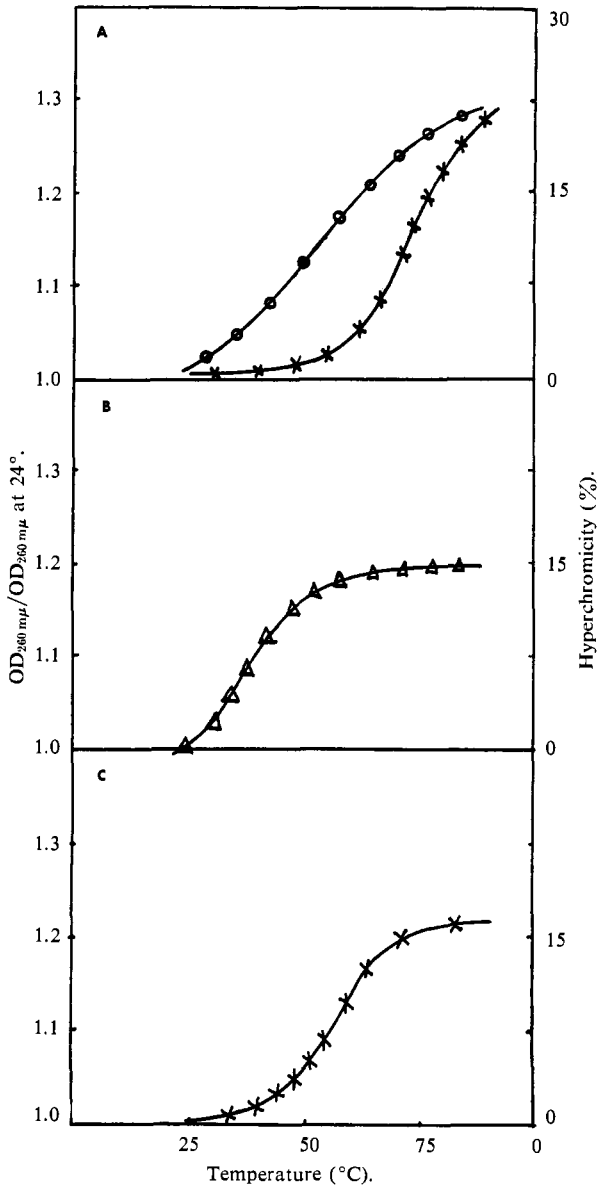

FIGURE 2: Thermal denaturation profiles. (A) Intact tRNA in 0.15 M NaCl (O–O–O) and in 0.15 M NaCl–0.005 M $MgCl_2$ (X–X–X). (B) Intact RNA in 7 M urea–0.005 M NaCl. (C) Major RNase digestion product (peak 1, Figure 3C) in 0.15 M NaCl–0.005 M $MgCl_2$.

Experimental Section

RNA Isolation. Mixed RNAs were isolated from growing yeast by a variation of the method of Monier *et al.* (1960) as described by Bell *et al.* (1964).

Physical Methods. Ultracentrifugal analyses were performed in 0.15 M NaCl using standard procedures. Molecular weight determinations were by the method of Ehrenberg (1957). Partial specific volume was determined from density measurements using the relationship[2]

$$\bar{v} = \frac{1}{d_0} - \frac{1}{x}\frac{(d - d_0)}{d_0}$$

where $\bar{v}$ = partial specific volume, d_0 = density of solvent, d = density of solution, and x = concentration of solute in grams per milliliter.

Digestion of RNA with Ribonuclease. RNA, at a final concentration of 1 mg/ml in 0.15 M NaCl–0.005 M $MgCl_2$, was digested with various concentrations of pancreatic RNase A (Worthington Biochemical Corp., Freehold, N. J.) for 30 min at 25°. After incubation, a one-tenth volume of 0.5% aqueous sodium dodecyl sulfate was added and mixed vigorously, and the mixture was extracted twice with equal volumes of 90% (w/v) phenol. The phenol extracts were washed with 0.15 M

[2] Schachman (1957).

NaCl–0.005 M $MgCl_2$ and the combined aqueous layers were extracted three times with equal volumes of peroxide-free ether. Residual ether was removed *in vacuo* at room temperature. It was found that no ribonuclease activity, as measured by the release of small oligonucleotides from RNA at 25°, was detectable after this treatment.

Gel Filtration. Partial digests of RNA were fractionated on Sephadex G-75 columns (2.5 × 35 cm) which had been previously equilibrated to the eluting solvents. The digests were applied in 2 ml and washed in with an equal volume of the eluting solvent. Flow rates of approximately 15 ml/hr were employed and all manipulations were carried out at room temperature except where otherwise stated.

Results

The isolated RNA was subjected to ultracentrifugational analysis and found to have the ollowing molecular parameters: sedimentation coefficient s_{20}(60 μg/ml) = 4.2×10^{-13} sec and s_{20}(7 mg/ml) = 3.8×10^{-13} sec; diffusion coefficient (during sedimentation) $D_s = 8.76 \times 10^{-7}$ cm²/sec and diffusion coefficient (without sedimentation) $D = 8.52 \times 10^{-7}$ cm²/sec.

The partial specific volume, $\bar{v}$, was found to be 0.55 (±3%) ml/g giving a molecular weight, for this RNA, of 25,400 (±5%) when substituted in the Svedberg equation $M = (RT/(1 - \bar{v}\rho))(S/D)$, where M = molecular weight, R = gas constant in ergs per degree per mole, T = temperature in °K, and ρ = density of solvent.

The thermal denaturation profiles for this RNA in 0.15 M NaCl and 0.15 M NaCl–0.005 M $MgCl_2$ are shown in Figure 2A.

Sephadex Fractionation of Partial Digests of RNA. The partial digests of RNA were applied to a Sephadex G-75 column which had been equilibrated to 0.15 M NaCl–0.005 M $MgCl_2$ and eluted with the same solvent. The elution profiles are shown in Figure 3B–E together with the profile of undigested RNA (Figure 3A). Sedimentation coefficients of peak fractions are also shown.

As can be seen from Figure 3B, limited treatment of RNA with RNase gives rise to two main products on Sephadex G-75 fractionation. These were classified by their positions on the column elution profiles as peak 1 (high molecular weight material) and peak 2 (low molecular weight material). Sedimentation coefficients were determined for various fractions through peak 1 and no significant variation from the value for intact RNA was observed. Rechromatography of this peak on Sephadex G-100, in the same solvent, gave no indication of the presence of more than one component. The peak 2 material, on the other hand, was shown by column chromatography on DEAE-cellulose (Cl^-) in the presence of 7 M urea (Tomlinson and Tener, 1962) to consist of oligonucleotides which were five nucleotides or less in length. When the RNase concentration was increased fivefold (Figure 3C), the material

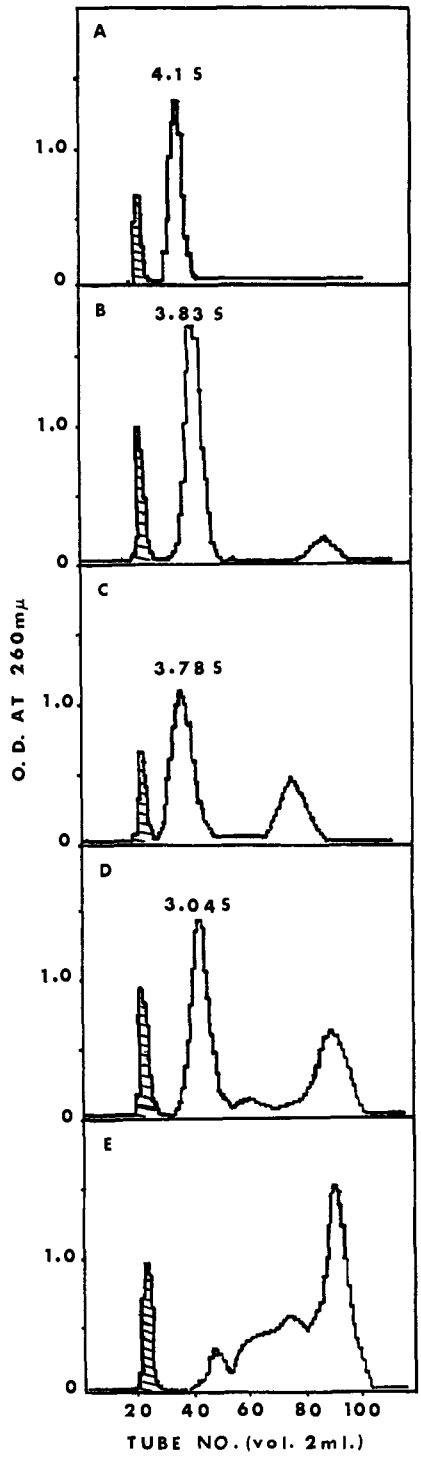

FIGURE 3: Elution profiles on Sephadex G-75 of RNA digested with pancreatic RNase. The hatched areas are blue dextran 2000, which is completely excluded from the gel. The eluting solvent was 0.15 M NaCl–0.005 M $MgCl_2$. (A) Undigested RNA. (B) RNA digested at a concentration of 0.5 μg of RNase/mg of RNA per ml. (C) RNA digested at a concentration of 2.5 μg of RNase/mg of RNA per ml. (D) RNA digested at a concentration of 5.0 μg of RNase/mg of RNA per ml. (E) RNA digested at a concentration of 20.0 μg of RNase/mg of RNA per ml. The s values of peak fractions are shown.

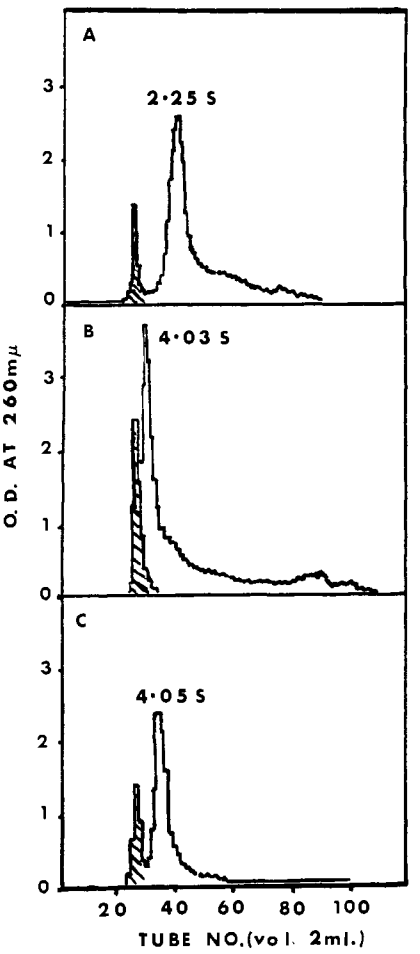

FIGURE 4: Elution profiles on Sephadex G-75 in the presence of 7 M urea. The hatched areas are blue dextran 2000. (A) Peak 1 material from RNA digested with 2.5 μg of RNase/mg of RNA per ml (Figure 3C), applied and eluted in 7 M urea–0.03 M Tris-HCl–0.005 M NaCl (pH 7.0) at 65°. (B) Intact RNA applied and eluted as in A. (C) Intact RNA applied and eluted in 7 M urea–0.03 M Tris-HCl–0.005 M NaCl (pH 7.0) at 20°. The *s* values quoted are those obtained after dialysis into 0.15 M NaCl.

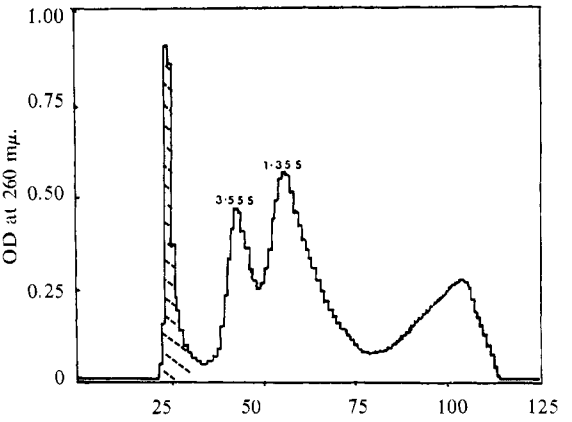

FIGURE 5: Elution profile on Sephadex G-75 of tRNA digested with 2.5 μg of RNase/mg of RNA per ml in 0.15 M NaCl–0.005 M $MgCl_2$ and then adjusted to 0.025 M EDTA at pH 7.0 before application. The eluting solvent was 0.15 M NaCl. The *s* values of major peaks are shown. The hatched area is blue dextran 2000.

of peak 2 constituted about 35–40% of the total nucleotide content. The characteristics of peak 1, however, remained virtually unchanged and a thermal denaturation profile in 0.15 M NaCl–0.005 M $MgCl_2$ (Figure 2C), although exhibiting a reduced T_m compared to that of intact RNA, revealed the continued existence of a high degree of secondary structure. A further twofold increase in RNase concentration (Figure 3D) still gave basically the same pattern but peak 1 showed a substantial decrease in *s* value. Another fourfold increase in RNase concentration caused extensive degradation with the production of a variety of new products (Figure 3E).

Gel Filtration in the Presence of 7 M Urea. In order to investigate the behavior of RNA under conditions unfavorable to the maintenance of secondary structure, intact RNA was chromatographed on Sephadex G-75 in 7 M urea–0.15 M NaCl–0.03 M Tris-HCl (pH 7.0) at room temperature. This gave an elution profile (Figure 4C) in which no change in RNA behavior could be detected (*cf.* Figure 3A). However, the thermal denaturation profile of RNA in 7 M urea (Figure 2B) revealed that, even in this solvent, the secondary structure of RNA was not completely destroyed until the temperature was raised to 65°. Intact RNA was, therefore, chromatographed on Sephadex G-75 in 7 M urea–0.15 M NaCl–0.03 M Tris-HCl (pH 7.0) at 65°. The resultant profile (Figure 4B) showed a dramatic change in that the RNA was almost excluded from the gel.

Peak 1 fractions, isolated after different levels of RNase digestion, were then chromatographed on Sephadex G-75 in 7 M urea–0.15 M NaCl–0.03 M Tris-HCl (pH 7.0) at 65°. The elution profiles gained from these experiments indicated that peak 1 had given rise to a new component and what appeared to be residual undigested RNA. It is important to note that no low molecular weight material was released by this treatment. As the concentration of RNase increased, this new component appeared in greater amounts until, at a level of 2.5 μg of RNase/mg of RNA per ml, no undigested RNA was detectable (Figure 4A). The sedimentation coefficient of this component, after the removal of urea by extensive dialysis against 0.15 M NaCl, was 2.25 S. On the other hand, undigested RNA, which had been treated in the same way, gave a sedimentation coefficient of 4.03 S.

Effect of EDTA. In order to investigate the role of magnesium, RNA was digested at 2.5 μg of RNase/mg of RNA per ml of 0.15 M NaCl–0.005 M $MgCl_2$ for 30 min at 25° as before and, after extraction with phenol and ether, was made 0.025 M with respect to EDTA at pH 7.0. This digest was then applied to a Sephadex G-75 column previously equilibrated with 0.15 M NaCl and then eluted with the same solvent. This resulted in a markedly changed elution profile (Figure 5) in which the RNA-like component had been replaced by two new components with sedimentation coefficients of 3.55 and 1.35 S. Intact RNA, treated in a similar manner, yielded an elution profile indistinguishable from that shown in Figure 3A.

Discussion

The most important conclusion which might be drawn from the above results is that the structure of RNA must be such that, at low RNase concentrations in the presence of Mg^{2+} ions, it is possible to remove up to 35–40% of the nucleotide content and still leave a residue (peak 1, Figure 3C) whose molecular parameters closely resemble those of intact RNA. This residue is, however, readily distinguishable from intact RNA by chromatography on Sephadex G-75 under conditions in which helix formation is prevented, *i.e.*, 7 M urea at 65° (see Figure 4).

Furthermore, when the urea has been removed by dialysis against 0.15 M NaCl, the residue has a sedimentation coefficient of only 2.25 S, whereas that of intact RNA is virtually unchanged. It therefore seems likely that this material mimics the properties of intact RNA by virtue of the continued existence in it of a great deal of the secondary and tertiary structure of RNA. This would be compatible with the considerable hyperchromicity which it exhibits when heated (Figure 2C). As is shown by the effect of EDTA (Figure 5), Mg^{2+} ions play an important role in the maintenance of this residual structure although the changes produced by EDTA clearly differ from those brought about by the complete destruction of hydrogen bonding.

However, as this study was not performed on a single RNA species, interpretation of the above results is complicated by the possibility that all RNA species may not be attacked by RNase at the same rate. If, for example, some species are much more susceptible than others, the limited action of RNase could produce a mixture of highly degraded and largely intact molecules, such that an RNA-like residue could appear to persist even after removal of about one-third of the nucleotides. If this were so, one would expect that even at very low levels of digestion substantial amounts of low molecular weight material would appear. In fact, even when very little low molecular weight material has been produced, a considerable amount of RNA has been converted to the RNA-like residue. Furthermore, rechromatography of the high molecular weight fraction, under conditions where secondary structure no longer exists, does not release any additional low molecular weight material. Therefore, although one cannot completely eliminate the possibility that a unique combination of reaction rates at various stages in the degradation might yield results similar to the above, it seems unlikely that some RNA species are rapidly destroyed while others remain virtually intact.

These findings, together with the fact that RNase almost certainly attacks single-stranded RNA regions preferentially, would, therefore, seem to indicate that RNA is comprised of a double-stranded structure with several single-stranded loops, the removal of which does not greatly alter the size or shape of the molecule. Of those models which have been proposed, the clover-leaf shape (Figure 1C, Holley *et al.*, 1965) seems best able to meet these requirements.

During the preparation of this paper similar experiments performed in part on a single RNA species have been reported by Wagner and Ingram (1966), on the basis of which a double-hairpin structure (similar to Figure 1A) was proposed (Armstrong *et al.*, 1966). However, the digestion conditions used (Litt and Ingram, 1964) differed from those reported above in several respects, making direct comparison of the data extremely difficult. The digestions were performed at 0° in the presence of 0.01 M $MgCl_2$ and in general higher RNase concentrations and longer incubation times were employed. Although, under certain conditions, this resulted in a release of low molecular weight material in quantities comparable to that shown in Figure 3C, the high molecular weight material which remained differed from that described above in that it consisted of a readily separable mixture of roughly 50% intact RNA and 50% of what were described as "half-molecules." The reasons for this important difference are not clear; however, it is important to note that these conditions of temperature (Henley *et al.*, 1966) and $MgCl_2$ concentration (Millar and Steiner, 1966) have been shown to cause considerable RNA aggregation. At 25° and 0.005 M $MgCl_2$, RNA aggregation appears to be insignificant.

It has recently been pointed out (Guschlbauer, 1966) that many of the known characteristics of RNA are explicable on the basis of models containing triple- rather than double-stranded helices. The models proposed consist basically of a single-hairpin structure (similar to Figure 1B), in which one leg is much longer than the other, enabling it to fold back and wrap around the existing double helix. As these structures were only described in the most general terms it is difficult to know how susceptible they might be to RNase attack or how well they could be made to fit the known nucleotide sequence of alanyl-tRNA or the complete sequences more recently established for tyrosinyl-tRNA (Madison *et al.*, 1966) and two seryl-tRNAs (Zachau *et al.*, 1966). Each of these four tRNAs can form a clover-leaf structure, resembling that shown in Figure 1C, such that certain important sequences (the anticodon, GpTpψpCpG, etc.) bear very similar relationships to each other and to the ends of the molecules.

A further piece of evidence which might also support

the clover-leaf hypothesis was reported by Henley *et al.* (1966) who found that between 20 and 40° (and to a large extent between 20 and 25°) RNA undergoes "a conformational change in which a small increase in hydrodynamic volume is compensated by a large decrease in asymmetry." This might well be due to the transition of the folded clover-leaf form shown in Figure 1D (Holley, 1966) into the open form shown in Figure 1C.

In summary it is felt that, although the possible existence of other RNA configurations, under certain conditions, cannot be ruled out, the available evidence best supports the clover-leaf model of RNA structure. Repetition of these experiments on single amino acid specific RNAs would, however, be more conclusive.

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