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Studies on the Secondary Structure of Phenylalanyl Transfer Ribonucleic Acid*

Alita Rosenfeld,† Charles L. Stevens, and Morton P. Printz

ABSTRACT: To investigate the secondary structure of *Escherichia coli* Phe tRNA the techniques of spectral analysis and tritium–hydrogen exchange were used on the reaction of 1% HCHO with Phe-tRNA at 35°. The rate and extent of the reaction of HCHO with Phe-tRNA is decreased in the presence of Mg^{2+} or high concentrations of Na^+. The tritium-exchange measurements were made to determine the amount of base pairing before, during, and after reaction. As expected, there is a decreasing number of slowly exchanging hydrogens upon increasing time of exposure to HCHO. Before reaction there were 84 H-bonded hydrogens in a 10^{-3} M Mg^{2+} solution and only 48 after reaction. The tritium exchange-out curve for Phe-tRNA in Mg^{2+}, treated with HCHO, coincided with unreacted Phe-tRNA in 0.03 M PO_4^{2-}. This suggested a stable "core" region of H bonding. The size of this core is that expected on the basis of a cloverleaf-like structure for tRNA.

The thermal denaturation curve of Phe-tRNA in 0.03 M phosphate shows a coincidence with the theoretical curve obtained using a statistical mechanical theory of thermal transitions on the cloverleaf model of Phe-tRNA. Other thermal denaturation and tritium-exchange experiments suggest that the Phe-tRNA molecule may have tertiary folding of the cloverleaf in 0.2 M Na^+ solution.

The primary structures of many tRNAs have been determined, and in all cases, models can be proposed in which the sugar phosphate chain is folded into a cloverleaf configuration (secondary structure). It has also been suggested that the arms of the cloverleaf then arrange themselves into a compact tertiary structure (Henley *et al.*, 1966). X-Ray crystallography of purified tRNAs should eventually deduce this tertiary structure. Kim and Rich (1969) suggest that the molecules are elongated and dimerized in an overlapping antiparallel fashion.

The reaction of formaldehyde with the amino and imino groups of the nucleic acid bases has been extensively used to study the secondary structure of RNAs. Fraenkel-Conrat (1954) showed that while most RNAs reacted with formaldehyde, DNA did not, probably because the bases are inaccessible in the double-helical configuration. The double-helical regions in RNA are thought to be shorter in length and therefore less stable and able to react with formaldehyde. Fasman *et al.* (1965) showed that the thermal denaturation curve of formylated tRNA resembles the broad curves of neutral

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poly(rA)[1] or poly(rC); in the absence of formaldehyde the denaturation of tRNA occurs over a much narrower range of temperature. This suggests that formylated tRNA is a single-stranded, base-stacked helix, while native tRNA has a higher degree of structure, *i.e.*, hydrogen bonding. Penniston and Doty (1963) showed that the presence of Mg^{2+} inhibits the rate and extent of reaction of formaldehyde with tRNA.

With the information gained from the studies of the reactions of poly(rA) (Stevens and Rosenfeld, 1966), and poly(rA:rU) (Stevens and Gal-Or, 1969), with formaldehyde, it was thought that some meaningful information on the secondary structure of Phe-tRNA could be elucidated using this reaction. It was found that the breaking of hydrogen bonds and addition of formaldehyde to the amino and imino groups of the bases could not be detected separately by measuring the change in optical absorbance at any specific wavelength or combination of wavelengths. The technique of tritium–hydrogen exchange gives the number of hydrogen bonds in a molecule and is unaffected by the addition of formaldehyde to the freed amino and imino groups. For this reason, the method of tritium–hydrogen exchange was used to monitor the formaldehyde reaction.

Hydrogens unhindered by structure exchange too quickly to be measured by the methods currently in use. Hydrogens involved in hydrogen-bonding exchange measurably slowly as has been shown in the cases of DNA (Printz and von Hippel, 1965), sRNA (Englander and Englander, 1965; Gantt *et al.*, 1969), and ribosomes (Page *et al.*, 1967). Printz and von Hippel (1965) have shown that the number of structural hydrogens measured in DNA corresponds closely to that predicted on the basis of two hydrogen bonds per adenine-thymine base pair and three per guanine-cytosine base pair.

We have determined the apparent number of hydrogen bonds present in the Phe-tRNA molecule at various stages in the formaldehyde reaction. In general, the number decreases with increased time of exposure to formaldehyde. Results suggest that Phe-tRNA in 0.03 M phosphate solution may be in a cloverleaf configuration.

Kallenbach (1968) developed a statistical mechanical theory of melting for low molecular weight polyribonucleotides capable of forming short regions of base pairs. The effects of helix size, loop size, and arrangement of base pairs are taken into account. Using the experimental melting curve of Felsenfeld and Cantoni (1964) for yeast serine tRNA in 0.01 M NaCl, Kallenbach observed agreement with this theoretical melting curve based on the cloverleaf model. This theory was also used to predict the melting curves for yeast alanine and tyrosine tRNA. However, the agreement between the theoretical and experimental melting profiles done in 0.2 M NaCl (Mahler *et al.*, 1963) is not good. An explanation for this is provided in this paper. Also presented is an argument which brings data on the melting curve of *Escherichia coli* Phe-tRNA in 0.03 M sodium phosphate buffer into agreement with Kallenbach's theoretical melting curve for the proposed cloverleaf structure.

[1] Abbreviations used are: poly(rA), polyriboadenylic acid; poly(rC), polyribocytidylic acid; poly(rA:rU), the two-stranded helical complex of the indicated homopolymers; THO, tritiated water; $Na\text{-}HPO_4$, sodium phosphate buffer consisting of the given molarity of the monobasic salt with sodium hydroxide added to adjust the pH.

Materials and Methods

Materials. The mixed *E. coli* tRNA was a gift from Dr. Harry Chen of Rockefeller University. Greater than 90% of the material had its terminal adenosine and was capable of accepting amino acids. The *E. coli* Phe-tRNA was a sample of the material produced at Oak Ridge National Laboratories in a project jointly sponsored by them and the National Institutes of Health. It was 65–70% pure (moles of phenylalanine accepted/mole of therminal adenosine). Phenylalanine acceptance of the stock material was 880 μμmole/optical density unit before experiments had begun and was 750 μμmole/optical density unit afterward. Phe-tRNA had an $s_{w,20}$ of 3.3 ± 0.1 both before and after a thermal denaturation experiment in 0.03 M phosphate buffer. Coarse Sephadex G-25 was purchased from A. B. Pharmacia. Tritiated water (1 Ci/g) was obtained from the New England Nuclear Corp. Reagent grade aqueous formaldehyde was distilled and stored in a light-tight glass bottle at 4°. Its concentration was determined by titration with sodium sulfite (Walker, 1953).

Spectrophotometric Measurements of the Formaldehyde Reaction. Absorbance measurements were made on a Cary Model 14M recording spectrophotometer equipped with the automatic sample changer accessory Model 1441. Stoppered 3-ml quartz cuvets with a 1-cm light path were sealed with silicone rubber. The temperature was controlled by circulating water from a Haake Model F thermostat to a cored cell block. A calibrated thermistor (Yellow Springs Instrument Co., No. 402) connected to a YSI Model 71 Thermistemp temperature controller was placed in a dummy cuvet and used to measure the temperature.

The solutions of tRNA were dialyzed against a 100-fold excess of 0.01 M EDTA (pH 7) and then three times against a 100-fold excess of the appropriate buffer. The solutions of Phe-tRNA were equilibrated in a 35° water bath. To each solution an appropriate amount of formaldehyde was added to give a final concentration of 1% and the resulting solution was mixed and put into a preheated cuvet. The adsorption spectrum between 320 and 220 nm was recorded at appropriate time intervals chosen to give a $\Delta OD_{260} \approx 0.004$. The cells were sealed with silicone rubber within 2 hr of the start of the experiment. When there was no further change in absorbance, the cuvets were removed and placed in a water bath which was subsequently heated to 90–95° for 0.5 hr. The solutions were allowed to equilibrate at 35° for at least 24 hr and a final absorption spectrum was recorded.

Tritium–Hydrogen Exchange of the Formaldehyde Reaction. The buffered solutions of tRNA at a concentration of 0.7–1.5 mg/ml were incubated at 35° and an appropriate amount of HCHO was added to give a 1% formaldehyde solution. At appropriate intervals, 1-ml samples of formylated tRNA were withdrawn and put in an ice bath. The tRNA solution was incubated 17–20 hr with tritiated water (final concentration 5–10 mCi/ml) at 0°. After 72–96-hr formylation a 1-ml sample was heated to 95° for 0.5 hr, put in an ice bath, and tritiated.

To measure the amount of tritium bound to the tRNA, one column exchange separation was used for time points of less than 5 min, two column runs for times of 8–15 min (Englander, 1963; Printz and von Hippel, 1968), and the rapid microdialysis technique of Englander and Crowe (1965) for exchange-out times of greater than 15 min. The ice-jacketed Sephadex columns were 1 × 10 cm and washed with the

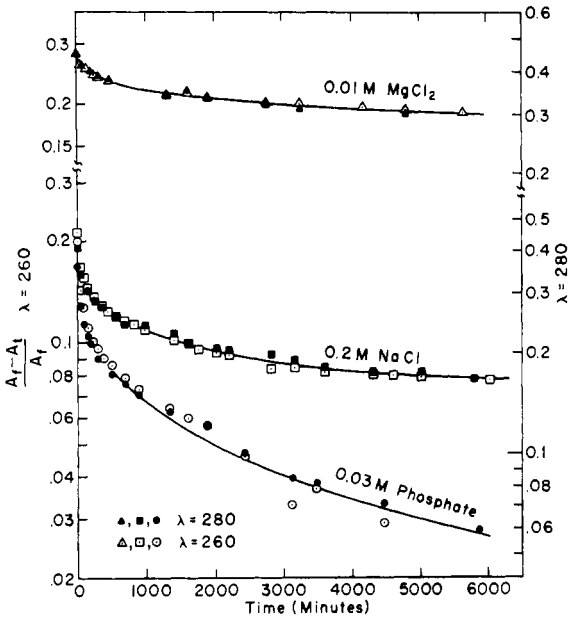

FIGURE 1: The kinetics of reaction of Phe-tRNA with 1% HCHO at 35°. The Mg^{2+} and 0.2 M NaCl solutions both had 0.001 M sodium phosphate (pH 7.4). A_t is the absorbance at time t and A_f is the absorbance after heating to 95° and reequilibration at 35°.

appropriate buffer. The procedures used followed those of Printz (1970) for two column and dialysis runs and Englander (1963) for one-column runs. In the one-column technique 0.02 ml of sample is layered on top of the column.

Because the molar extinction coefficient of tRNA depends on the extent of formylation, a control consisting of a known concentration of tRNA and an appropriate amount of HCHO was included in all experiments. Thus, the absorbance of experimental samples could be converted into concentration of tRNA.

The number of exchangeable hydrogens per molecule is determined as described by Englander and Englander (1965). The molar extinction coefficient, E_m, used for mixed tRNA with high Na^+ or Mg^{2+} present was 5.41×10^5 cm^{-1}. This is based on mixed tRNA at a concentration of 1 mg/ml having an OD_{260} of 21.4 and a molecular weight of 25,300. Mixed tRNA in 0.03 M sodium phosphate has an E_m value of 5.61×10^5 cm^{-1}. *E. coli* Phe-tRNA has a molecular weight of 27,000 and therefore, its E_m value is 5.85×10^5 cm^{-1} in Mg^{2+} or high Na^+ solution and 6.06×10^5 cm^{-1} in 0.03 M sodium phosphate.

In general, with careful technique, the probable error of any measured point on an exchange curve is less than 5%. At the extremes of the curve, the error may be as high as 10%. The overlapping of the exchange curves in Figures 2 and 3 demonstrates that the lower error limit was not exceeded in duplicate experiments. Because of a lack of material, we were unable to repeat the Phe-rRNA exchange experiments. Therefore, we must assume 10% as the probable error of any point, although the actual error is less.

The number of slowly exchangeable hydrogens was obtained from the intersection of the exchange curves with the zero-time ordinate. For this purpose we made a linear extrapolation from the earliest time points. Recent data indicate that there are observable, exchanging hydrogens which exchange much faster than those shown in the figures in this paper. These are seen when the earliest time points are 10–20 sec (Englander *et al.*, 1970; M. P. Printz, personal communication). In this paper the extrapolation point is determined from the slowly exchanging hydrogens, with rate constants equal to or less than the rate constants observed in studies of structured DNA (Printz and von Hippel, 1965). The latter have been shown to arise undoubtedly from hydrogen-bonded hydrogens. Thus, our extrapolation point is a measure of those hydrogens most likely involved in structurally stabilized hydrogen bonds.

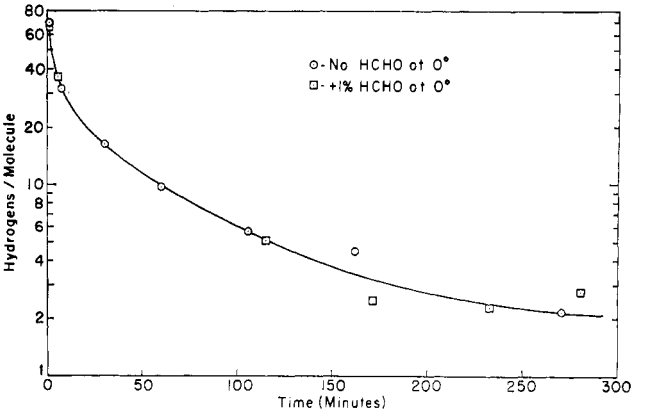

FIGURE 2: Exchange-out curve of mixed tRNA in 0.03 M sodium phosphate buffer. One sample was exposed to 1% HCHO during the incubation with THO for 17 hr at 0°; the other was not.

Thermal Denaturation Experiments. The cuvets, filled with tRNA solution, were sealed with silicone rubber and placed in a cored cell holder through which water was circulated by a Haake Model F thermostat. The temperature was measured as described above. The solutions were heated in steps and the absorption spectrum from 320 to 220 nm was recorded after the absorbance no longer changed. Absorbances were corrected for expansion of water.

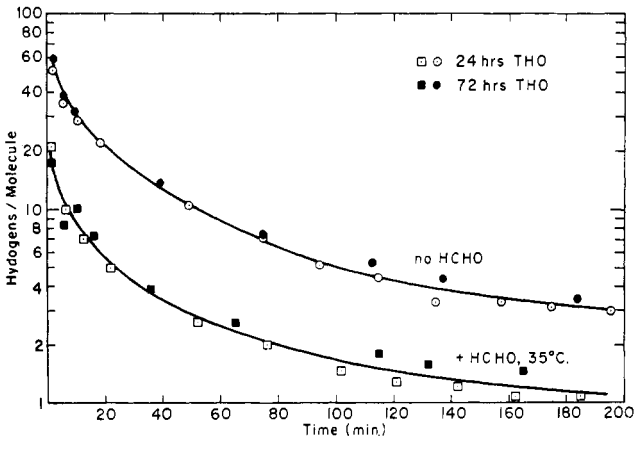

FIGURE 3: Exchange-out curves of mixed tRNA in 0.03 M sodium phosphate buffer. The circles represent the exchange-out kinetics when the tRNA was exposed to THO at 0° for the indicated lengths of time. The squares represent the exchange points of the tRNA after incubation in the 1% HCHO for 72 hr at 35° before incubation with THO at 0° for the indicated lengths of time.

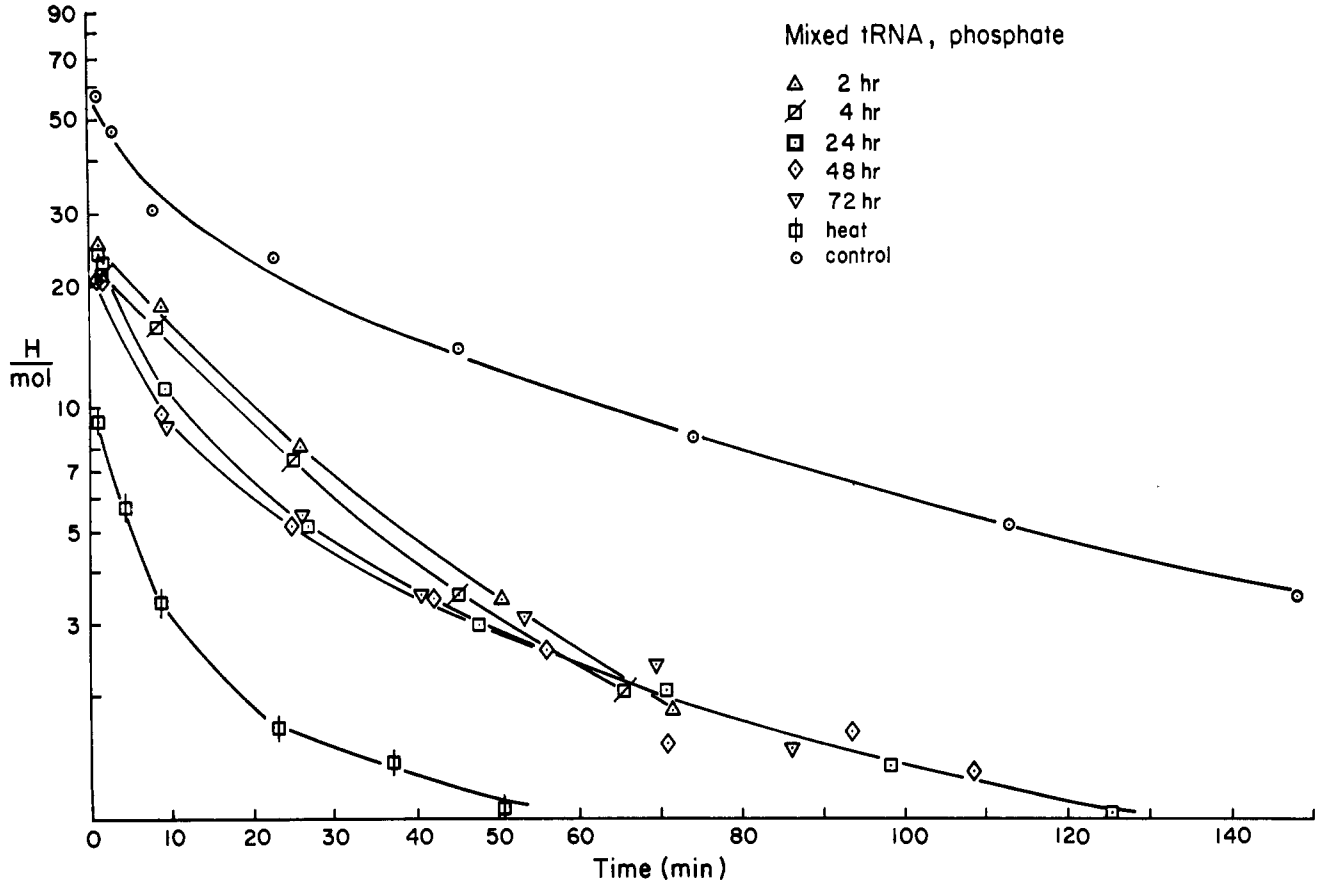

FIGURE 4: The exchange-out curves of mixed tRNA in 0.03 M sodium phosphate buffer during the reaction with 1% HCHO at 35°.

Results

Spectrophotometric Kinetics of Formaldehyde Reaction. The reaction of formaldehyde with nucleic acids involves a collapse of hydrogen-bonded base pairs and reaction with the freed bases. Both of these reactions result in an increase in the extinction coefficient of the nucleic acid. In certain cases, it has been possible to measure these reactions independently by a careful selection of wavelength for the kinetic measurements.

TABLE I: Extent of Formaldehyde Reaction.[a]

	λ = 260 nm			λ = 280 nm		
	0.03 M	0.2 M Na^+	0.01 M Mg^{2+}	0.03 M	0.2 M Na^+	0.01 M Mg^{2+}
$\frac{A_p - A_o}{A_t - A_o}$	0.861	0.663	0.323	0.838	0.631	0.342
$\frac{A_t - A_p}{A_t - A_o}$	0.139	0.337	0.677	0.162	0.369	0.658

[a] A_o is the absorbance before the formaldehyde reaction (corrected for dilution), A_p is the absorbance after 100-hr reaction at 35°, and A_t is the absorbance after heating to 95° and reequilibration at 35°.

The reaction of *E. coli* Phe-tRNA with 1% HCHO at 35° was followed spectrophotometrically at 260 and 280 nm. The results are shown in Figure 1. By the displacement of the ordinate axis it is seen that the kinetics of the reaction are the same for 260 and 280 nm at identical salt conditions. The lack of dispersion in the absorption kinetics of the formaldehyde reaction can mean that few A-U base pairs are broken (since the absorption at 260 nm would be affected while at 280 nm it would be unaffected) and/or such a complex overlap of reactions occurs that the effect of A-U base pairs breaking would be masked by the effects of the other reactions.

The extent of the formaldehyde reaction with Phe-tRNA under various conditions is shown in Table I. There is a decreasing extent of reaction with an increase in Na^+ concentration or addition of Mg^{2+}. The decreased extent of formaldehyde reaction upon addition of Na^+ or Mg^{2+} indicates that these ions stabilize the hydrogen bonding of the nucleotide pairs and prevent the reaction of some of the nucleotides with formaldehyde. In all cases, the reaction is not completed until the tRNA is heated to 95°.

Tritium–Hydrogen-Exchange Experiments. Hydrogen exchange is used here as a probe of the reaction of formaldehyde with tRNA. The spectral measurements detect both the actual combination of HCHO with a base and the disruption of secondary and tertiary structure. Hydrogen exchange allows for a more direct measure of the structural perturbations which occur during the formaldehyde reaction.

It was found that incubation of the THO with the tRNA

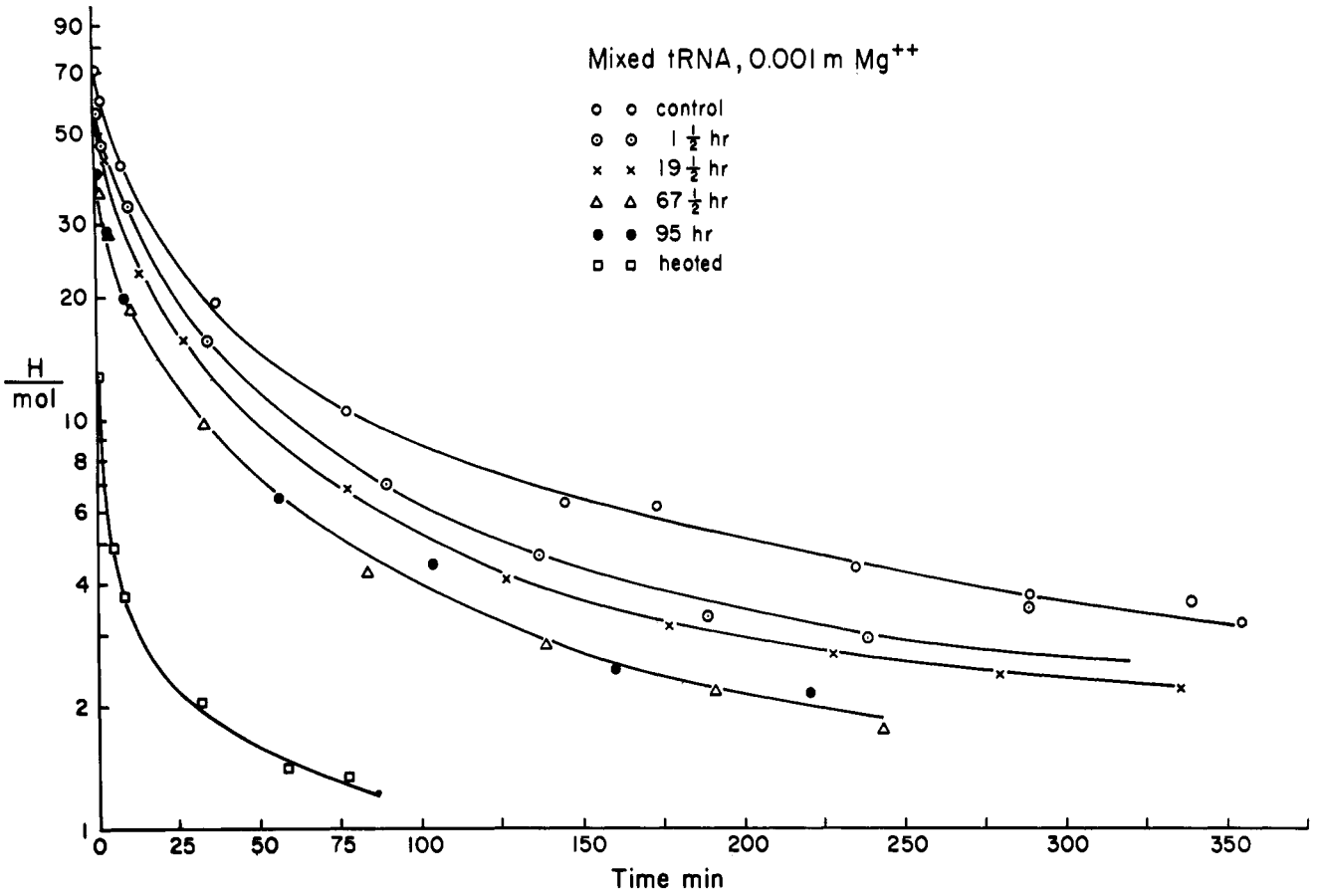

FIGURE 5: Same as Figure 4 except 0.001 M $MgCl_2$ was added to the solution.

at 35° would result in an increase in the number of exchangeable hydrogens with increasing time of incubation at 35°. This was attributed to anomalous exchange of the C-8 hydrogen on the purines (Shelton and Clark, 1967). It is seen in Figure 2 that 1% HCHO at 0° for 17 hr will not cause any decrease in the number of hydrogen bonds in tRNA. In Figure 3 it can be seen that the THO reaches equilibrium with the exchangeable hydrogens of tRNA in 24 hr at 0° both before and after reaction with HCHO. These control experiments show that the procedures used to determine the extent of hydrogen bonding in tRNA during formaldehyde reaction at 35° were valid.

A series of exchange-out curves of mixed tRNA in 0.03 M Na-PO_4 buffer at increasing times of exposures to 1% HCHO at 35° are shown in Figure 4. Those curves with 10^{-3} M Mg^{2+} added to the buffer are shown in Figure 5. Because of the limited amount of Phe-tRNA available for all experiments (10 mg) only a limited number of exchange-out curves of Phe-tRNA during HCHO reaction could be done. Figure 6 shows the results for Phe-tRNA in phosphate buffer and Figure 7 the results when 10^{-3} M Mg^{2+} is present. In each set of curves is included an exchange-out curve of a tRNA sample before HCHO addition and a sample of tRNA which had been heated to 95° with HCHO present after reaction at 35°.

The nature and extent of the formaldehyde reaction with tRNA is clearly shown in the tritium–hydrogen-exchange data. The loss in the total number of slowly exchangeable H-bonded hydrogens (*i.e.*, the zero-time extrapolation point) with time of exposure to formaldehyde is evident. When Mg^{2+} is present in the tRNA solutions there is an increase in the number of slowly exchanging hydrogens of tRNA both before and after reaction with HCHO. This is seen in spectrophotometric experiments also, with $(A_f - A_t)/A_f$ being higher when Mg^{2+} is present both before and after reaction at 35° (Figure 1). That there are differences between mixed and Phe-tRNA solutions is also evident. In 0.03 M sodium phosphate practically all the potentially labile H-bonded hydrogens of mixed tRNA are denatured after 2-hr reaction with HCHO. In Phe-tRNA the potential reaction was hardly started in that time. The final extent of the reactions also differed, with mixed tRNA having only 20 H-bonded hydrogens remaining while Phe-tRNA has almost twice as many. This suggests that Phe-tRNA has regions of hydrogen-bonded structure which are more stable than the average tRNA molecule.

The exchange-out curves for unreacted Phe-tRNA in 0.03 M $NaHPO_4$ and Phe-tRNA with Mg^{2+} and 1% HCHO present for 97 hr are shown together in Figure 8. The coincidence of the experimental points is apparent. This indicates that 45–50 of the H-bonded hydrogens are in a "stable" configuration.

Thermal Denaturation Experiments. The statistical mechanical theory of Kallenbach (1968) seeks to explain the thermal denaturation profile of tRNA in terms of the "cloverleaf" model of those species whose primary structure is known.

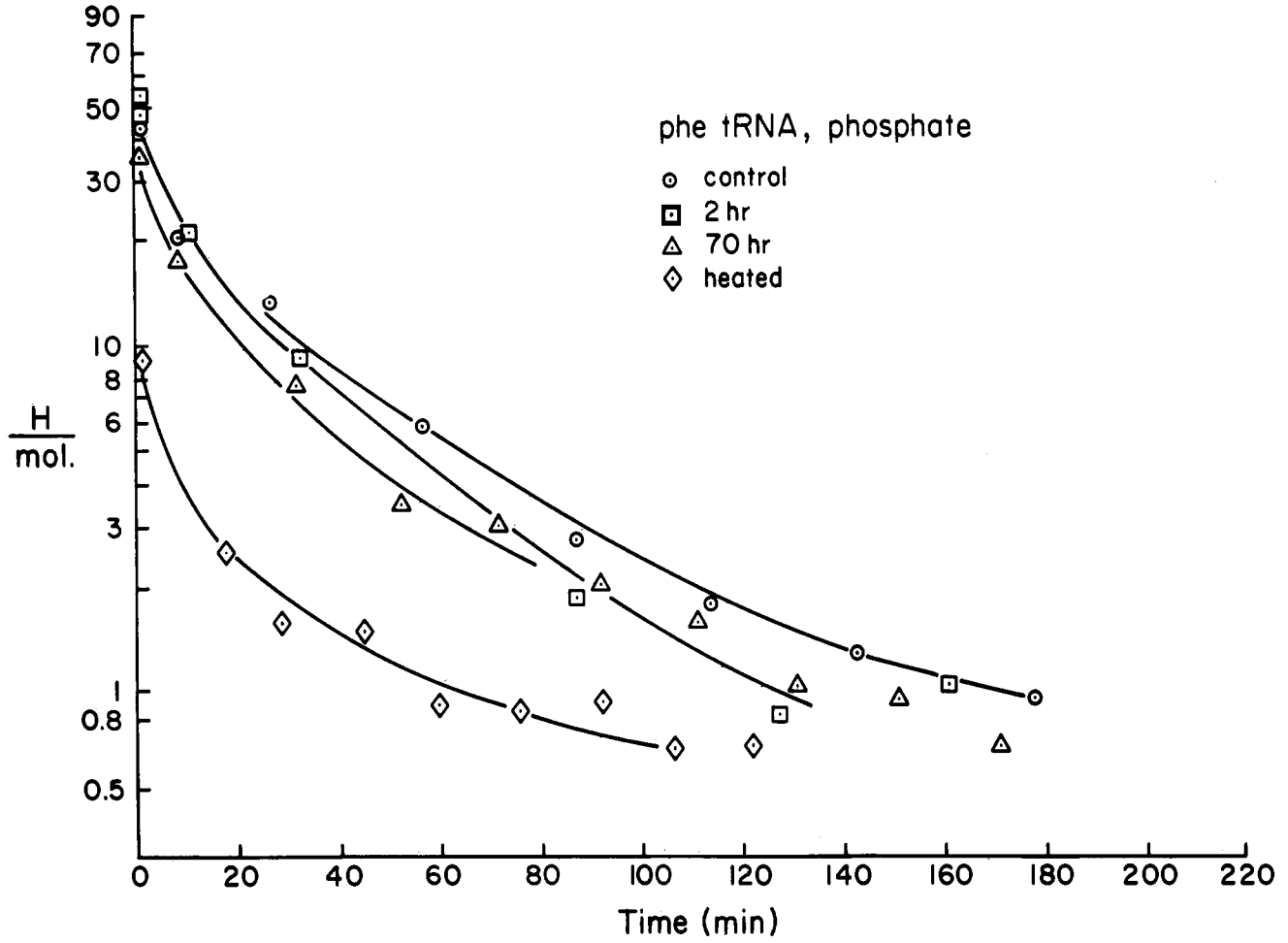

FIGURE 6: Exchange-out curves for Phe-tRNA in 0.03 M sodium phosphate buffer (pH 7.4) during the reaction with 1% HCHO at 35°.

The points of Figure 9 are calculated from experimental measurements of the thermal denaturation of Phe-tRNA at the ionic conditions and wavelengths indicated. All absorbancies were corrected for volumetric expansion. Fresco *et al.* (1963) showed that at 272.5 nm the change in molar extinction coefficient for the disruption of an A-U base pair is the same as a G-C pair. The experimental points were adjusted to account for a 3% increase in absorbance over the region 0–25°. The error bars indicate the possible error if $A_T - A_{25°}$ is increased 0.002 of an optical density unit while $A_{95°} - A_{25°}$ is decreased 0.002 of an optical density unit and *vice versa*. Because of the correction for volume expansion the error bars increase in length with increasing temperature.

The solid line represents the thermal denaturation curve calculated from Kallenbach's theory, shifted 14° lower, for the model shown in Figure 10 with all arms hydrogen bonded except IIa and only one A-U base pair at the end of arm III. The short-dashed line represents the calculated curve, shifted 18° lower, for all arms hydrogen bonded except IIa and IIb and three A-U base pairs at the end of arm III. The long-dashed line is the calculated curve, shifted 23° lower, of the model shown in Figure 11 with all arms hydrogen bonded except II. This second model for *E. coli* Phe-tRNA is reported by Barrell and Sanger (1969) using a tRNA sample prepared differently from that used by Uziel and Gassen (1969) and us.

The solid lines in Figure 12 are the theoretical transition curves for the four individual loop–helix units in the structure for Phe-tRNA shown in Figure 10. The dashed line is the curve for arm II of the model of Phe-tRNA shown in Figure 11. Arm II of the model in Figure 10 is certainly the least stable arm.

Discussion

When the experiments with Phe-tRNA were begun it was hoped that the collapse of hydrogen-bonded base pairs and reaction of free bases with formaldehyde could be measured independently. Other laboratories make use of the difference spectra between RNA reacted with HCHO at room temperature and unreacted RNA at high temperature to determine a wavelength that is insensitive to formaldehyde reaction (Doty *et al.*, 1959; Boedtker, 1967; Boedtker and Kelling, 1967). However, formylated RNA at 15–25° is not a random coil, as RNA at high temperature is; rather it is most probably a structure with both single-stranded base stacking and some hydrogen-bonded base pairs. Evidence for this conclusion is that upon heating there is a signficant increase in absorbance (Boedtker and Kelling, 1967), and also the residual hydrogen bonds found after heating by hydrogen–tritium exchange in Figures 4–7 above. Also, after reaction with formaldehyde,

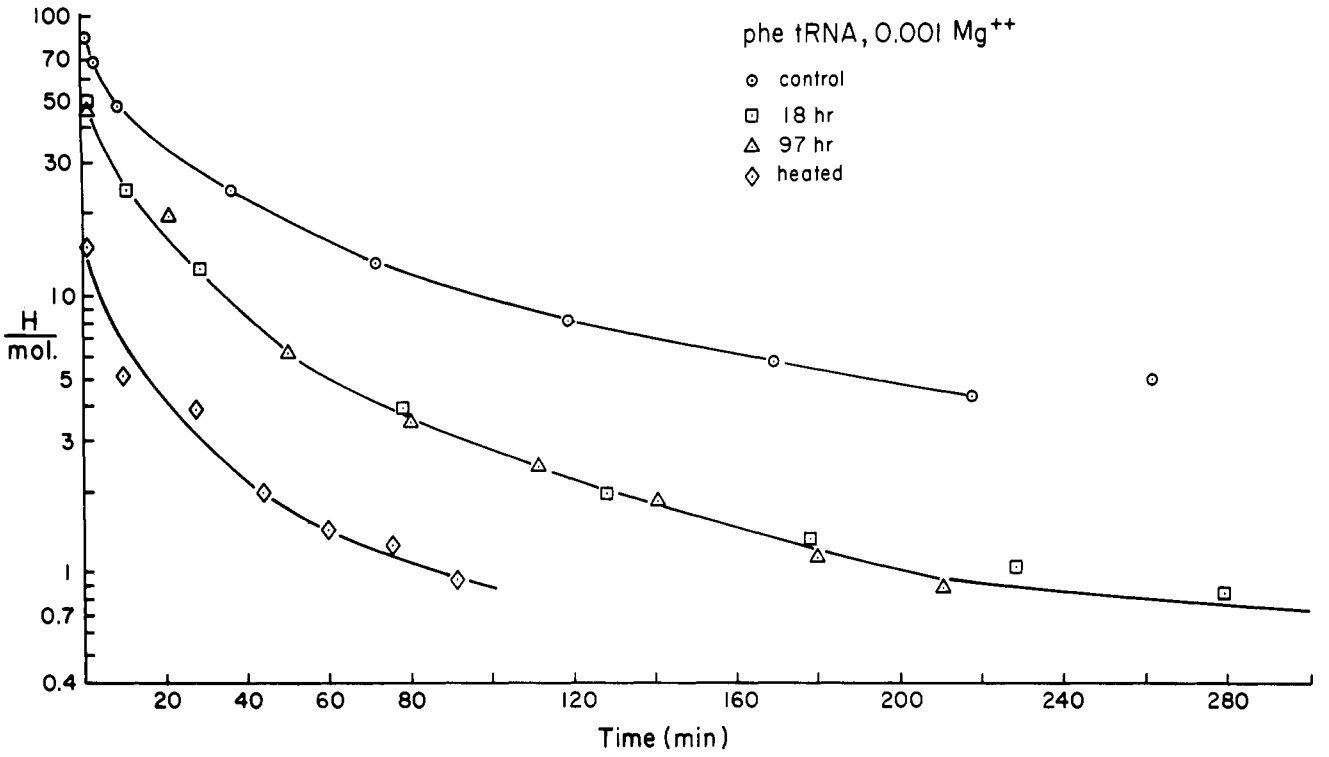

FIGURE 7: Same as Figure 6 except 0.001 M $MgCl_2$ was added to the buffer.

poly(rA) (Stevens and Rosenfeld, 1966) and poly(rC) (Fasman *et al.*, 1964) both exhibit hyperchromism upon heating due to the unstacking of the bases. Therefore, it is impossible to obtain a difference spectrum for natural RNAs that will show what wavelength, if any, is insensitive to formaldehyde addition.

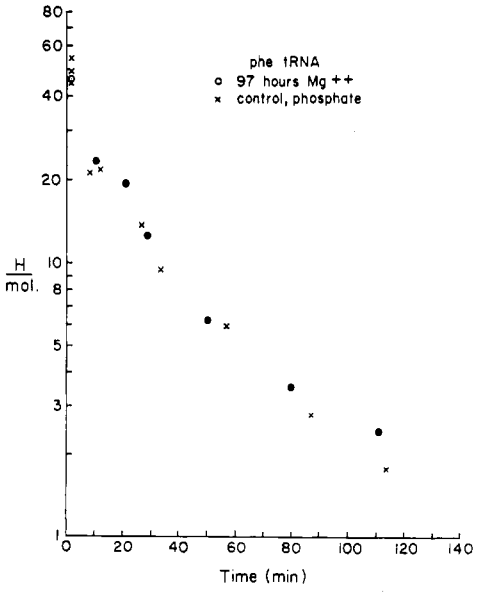

FIGURE 8: The exchange-out points for unreacted Phe-tRNA in 0.03 M sodium phosphate buffer and Phe-tRNA with Mg^{2+} and 1% HCHO present for 97-hr reaction at 35°.

In Phe-tRNA the regions of proposed base pairing are very short and at least in the 0.03 M $NaHPO_4$ solution the Phe-tRNA has many free bases ready to react with formaldehyde without any base pairs being denatured. As shown in Figure 1, the kinetics of the reaction of formaldehyde with Phe-tRNA are the same for 260 and 280 nm. The absorbance kinetics, therefore, reflect the reaction of formaldehyde with

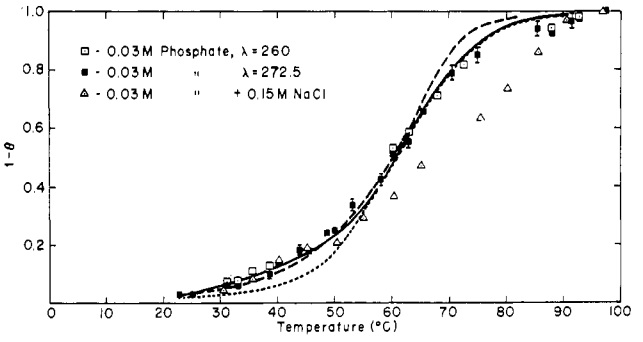

FIGURE 9: Comparison of experimental and theoretical transition curves for Phe-tRNA. The solid line represents the $1 - \theta$ (fraction denatured) transition curve for the model in Figure 10 with all arms H bonded except IIa and only one A-U base pair at the end of arm III. The short-dashed line represents the calculated curve for all arms H bonded except IIa and IIb and three A-U base pairs at the end of arm II. The long-dashed line is the calculated curve of the model shown in Figure 11 with all arms H bonded except II. The experimental points are a plot of the parameter $(A_T - A_{25°})/(A_{95°} - A_{25°})$ with the absorbancies corrected for volumetric expansion. A_T is the absorbance at temperature T, $A_{25°}$ is the absorbance at 25°, and $A_{95°}$ is the absorbance at 95°.

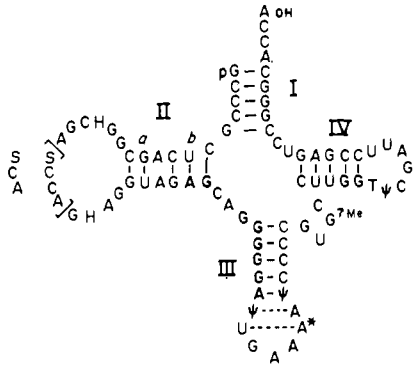

FIGURE 10: The cloverleaf model of phenylalanine tRNA from *E. coli* B as proposed by Uziel and Gassen (1969). Abbreviations of rare bases: H, 5,6-dihydrouridine-3′-PO_4; ψ, pseudouridine-3′-PO_4; *A**, 2-thiomethyl-6-isopentenyladenosine-3′-PO_4; G^{7Me}, 7-methylguanosine-3′-PO_4.

the free bases, the denaturation of base pairs, and the reaction of HCHO with these released nucleotide residues. On the other hand, exchange detects the labilization of interbase hydrogen bonding as a result of the formaldehyde reaction. Thus, exchange allows for a more direct measure of the structural perturbations which occur during the reaction.

The formaldehyde reaction in the presence of Mg^{2+} leaves a major fraction of the interbase hydrogens in a stable configuration. This fraction, we suggest, makes up a core region of the molecule whose structure is invariant with changes (within limits) in the solvent environment. For example, approximately the same number of measureable hydrogens with similar kinetics exists in the absence of Mg^{2+} prior to any formaldehyde reaction. If this interpretation is correct, then we would suggest that this core region may be the hydrogen-

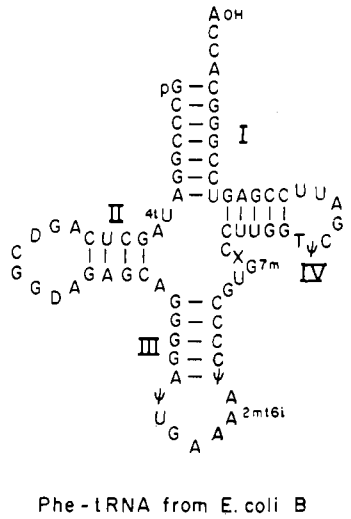

FIGURE 11: The cloverleaf model of Phe-tRNA from *E. coli* B as proposed by Barrell and Sanger (1969). Abbreviations of rare bases: D, dihydrouridine-3′-PO_4; ^{4t}U, 4-thiouridine-3′-PO_4; ψ, pseudouridine-3′-PO_4; A^{2mt6i}, 2-thiomethyl-6-isopentenyladenosine-3′-PO_4; G^{7m}, 7-methylguanosine-3′-PO_4; X, unknown.

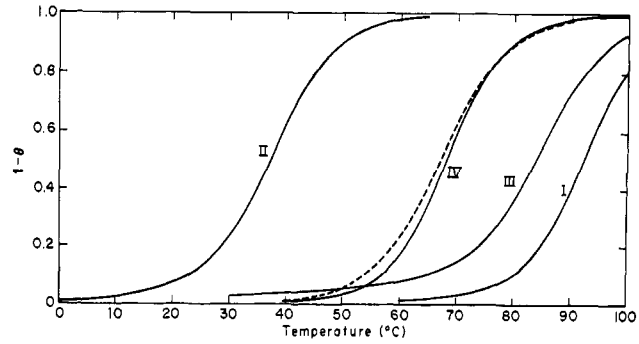

FIGURE 12: Theoretical transitions for the individual arms of the proposed cloverleaf models. The solid lines represent the indicated arms of the model in Figure 10. The dotted line represents arm II of the model shown in Figure 11.

bonded configuration of the tRNA molecule present in cloverleaf models. The additional measureable hydrogens, observed in the presence of Mg^{2+}, is attributed to the formation of the tertiary structure of the molecule.

The tritium–hydrogen-exchange experiments show that there is always a small residue of slowly exchanging hydrogens after heat treatment. It was expected that heating the samples to 95° would destroy any remaining base pairing and the presence of formaldehyde would prevent its reformation. One explanation for these base pairs is that the formaldehyde reaction does not go to completion with the nucleotides. The equilibrium constants between the formylized and unformylized bases range in value from 3 to 10 and this would result in 23–50% of the bases being unformylized at any time. Stable base pairs could then form between the unformylized bases. Also, the HCHO reaction can be reversed if base pairing is stable enough.

Thermal Denaturation Experiments. Kallenbach (1968) has developed a statistical mechanical theory of thermal transitions in short polynucleotides which achieve their secondary structure primarily by intramolecular complementary base-pair interactions. Chief examples are the various species of tRNA. Kallenbach uses a theoretical approach already applied somewhat successfully to thermal transitions in DNA (Crothers *et al.*, 1965; Crothers and Kallenbach, 1966). Polynucleotides such as tRNA, however, present some new difficulties. The theory requires a knowledge of the number of helical regions in the molecule, the specific sequence of A-U and G-C base pairs in each region, and the number and size of loops containing nonbonded residues. Such information is usually provided by hypothetical structures of tRNA species when the nucleotide sequence is known. The well-known "cloverleaf" structures are examples. It has been extremely difficult to show experimentally whether or not such hypothetical structures actually exist in the molecule. The Kallenbach theory can be of some use in deciding if such structures are at least consistent with the thermal denaturation profiles of the corresponding tRNA species.

At its present stage of development, however, the theory cannot be considered comprehensive because some relevant effects are not taken into account or are incompletely treated. These effects together with the general limitations of the theory are discussed extensively by Kallenbach. We wish to discuss briefly here one of the limitations which has a bearing

on the treatment of our data. The theory recognizes explicitly the difference in intrinsic stability of A-U and G-C pairs, symbolized s_A and s_G, respectively. Kallenbach assumes a direct proportionality between s_A and s_G and evaluates the proportionality constant by noting the linear dependence of T_m on G-C content of many double-helical RNA compounds, including poly(rA:rU), poly(r(AU)), and poly(rG:rC), in 0.2 M salt solution. It is desirable, however, to compare theory to experiments performed with solutions of different salt concentrations.

It can be argued that changes in ionic strength affect mainly the T_m of the transition and not its breadth. The degree of conversion from helix into coil is usually expressed in terms of the helix initiation parameter, σ, and the helix propagation parameter, s, referred to above. The initiation parameter, σ, arises largely from the stacking interactions of adjacent base pairs. It is believed that these interactions arise from dipole–dipole interactions which are not dependent on ionic strength. This expectation is borne out with polyadenylic acid in solution of neutral pH for which base-pairing interactions are absent. The stacking is found to be independent of ionic strength over a wide range (Leng and Felsenfeld, 1966). Probably there are other contributions to σ, but these should be small. Moreover, Kallenbach points out that the form of the theoretical transitions are quite insensitive to the value of σ used in the computation.

The parameter s is given by the integrated form of the van't Hoff equation: $\ln s = \Delta H(1/T_m - 1/T)/R$. The process here is the formation of 1 mole of helix segments (either A-U or G-C) from coil segments when end effects are negligible. There is little information in the literature on the dependence of ΔH on ionic strength. For poly(rA:rU), Ross and Scruggs (1965) found ΔH to be the same in 0.1 and 0.5 M sodium chloride solutions (-6500 ± 100 and -6685 ± 100 cal per mole, respectively, at 37°). To the extent that such independence is at least approximated for A-U and G-C pairs in RNA, it can be concluded that ionic strength affects s mainly through T_m and therefore will not have a large effect on the breadth of the transition. This expectation is upheld in thermal denaturation experiments with poly(rA:rU); the shape of the denaturation profile is the same whether the experiment is carried out with the polynucleotide in 0.06 or 0.11 M NaCl solutions (C. L. Stevens, 1969, unpublished data).

The point to be made is that, if the theory of Kallenbach can be used to compare theory and experiment, it is the breadth (and shape) of the transition profiles that should be compared and not the respective T_m. Kallenbach points out that the breadth is quite sensitive to the number, nature, and sequence of base pairs within a helical region. He computed a theoretical transition profile for yeast serine tRNA using the cloverleaf structure of Zachau *et al.* (1966) and compared it with the experimental profile obtained by Felsenfeld and Cantoni (1964). The theoretical profile had to be shifted to lower temperatures because the data were for tRNA in solution containing 0.01 M sodium ion (not 0.2 M). With this shift, the theoretical and experimental profiles were superimposable. Kallenbach also reported results for yeast alanine and tyrosine tRNA based upon data of others. Although solutions of RNA about 0.2 M in sodium ion were used, the agreement was not good: in both cases the observed transitions were broader than the predicted ones. Below, we discuss the possibility that the experimental transitions were too broad because in these solutions with rather high salt concentration, there was more extensive base pairing than would be predicted on the basis of proposed cloverleaf structures.

"One column" tritium–hydrogen-exchange experiments were done with Phe-tRNA in 0.03 M $NaHPO_4$ with 0.15 M NaCl added. They yielded 79.7, 77.9, 78.5, and 68.2 H-bonded hydrogens per molecule for exchange times of 46, 75, 77, and 106 sec, respectively. This number of hydrogen bonds is much higher than would be expected from the proposed cloverleaf models. For example, the cloverleaf structures in Figures 10 and 11 predict 55 and 58 hydrogen bonds, respectively, while the exchange experiments indicate 79 H bonds for the molecule in 0.2 M Na^+ and 84 H bonds in Mg^{2+} solution. These additional hydrogen bonds may be attributed to a structure more extensive than the cloverleaf, *i.e.*, tertiary folding of the cloverleaf.

Other experimental evidence shows that tRNA in high Na^+ has a more extensive structure than indicated in low salt solutions. Henley *et al.* (1966) found two phases to the denaturation of tRNA in 0.2 M Na^+. Between 20 and 40° there occurs a substantial conformational change, indicated by changes in sedimentation and viscosity with only a small increase in absorbance. The small increase in absorbance is also seen in the data in Figure 9. Above 40° the parameters increase in a coincident manner. On the other hand, Millar and Steiner (1966) show that the optical rotation, viscosity, and absorbance thermal profiles are coincident between 30 and 70° for tRNA in 0.01 M potassium acetate.

We may speculate as to which of the proposed cloverleaf models best fits the data presented here. Cloverleaf configurations with 45–50 H-bonded hydrogens were used in calculating the theoretical thermal transition profiles of Figure 9. This is the number of H-bonded hydrogens indicated by the hydrogen-exchange data in Figure 8 as being in a stable configuration. The experimental data fit the solid line in Figure 9 slightly better than the other two lines. The double-helical section IIa is certainly the least stable of the arms in Figure 10. The long-dashed line derived from Barrell and Sanger's model (Figure 11) assumes the hydrogen-bonded region in arm II is not stable in 0.03 M sodium phosphate. However, it is seen in Figure 12 that arm II would denature at almost the same temperature as arm IV. This indicates that they have the same stability, and therefore it would be hard to find conditions where arm II would not be hydrogen bonded while arm IV was hydrogen bonded. The third configuration tested (short-dashed line) has only three unpaired bases in the loop of arm III. While this is possible, the model of the anticodon loop proposed by Fuller and Hodgson (1967) requires seven unpaired bases and almost all other proposed cloverleaf configurations have seven unpaired bases in the anticodon loop. The theoretical denaturation curve, shifted 21° lower, of the intact cloverleaf model depicted in Figure 11 coincides, in Figure 9, with the long-dashed line at higher temperatures and the short-dashed line at lower temperatures. The calculation on the intact cloverleaf model of Figure 10 was not done, since this type of structure is not covered in this theory.

Mg^{2+} is necessary for the biological functioning of tRNA. Almost all physicochemical parameters which reflect the secondary and tertiary structure of tRNA change when Mg^{2+} is added to the buffered solution. The data presented here support the proposed cloverleaf-like structures for tRNA

and indicate that tRNA in Mg^{2+} solution has additional hydrogen bonding which might help in maintaining the compact tertiary structure necessary for biological activity. This additional hydrogen bonding could be between regions which are already hydrogen bonded or between bases in the loops which are brought into proximity by a folding of the arms over one another. Levitt (1969) proposed a general model for tRNAs which contained, in addition to the cloverleaf hydrogens, 25 additional H-bonded hydrogens involved in the tertiary structure. While we cannot demonstrate the validity of his model, we would point out that these additional hydrogens are in agreement with the number of extra slow hydrogens that we observe in the presence of Mg^{2+}.

While the data presented here indicate tertiary structure for tRNA in both high sodium and magnesium solutions, we have no evidence indicating that these tertiary structures are the same. That different tertiary structures may exist for certain tRNA molecules in Mg^{2+} solution, one biologically active and the other inactive, has been demonstrated by both Fresco *et al.* (1966) and Sueoka *et al.* (1966).

While we have interpreted our data in terms of published cloverleaf structures for Ph-tRNA and, under some conditions, a further tertiary folding of the molecule, we wish to point out that our data do not necessarily exclude other structural models. We have shown that, by both the method of tritium–hydrogen exchange and thermal denaturation, such a model is semiquantitatively consistent with the data. Alternate models consisting of about the same nature and extent of intramolecular H bonding perhaps would do as well.

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