Rate of Tritium Labeling of Specific Purines in Relation to Nucleic Acid and Particularly Transfer RNA Conformation†

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ABSTRACT: The kinetics of the incorporation of tritium into the C-8 positions of purine units in nucleic acids has been studied. The polymers investigated include poly(A), poly(A):poly(U) duplex, a double-stranded viral RNA, tRNA, and DNA. In the random coil state, the kinetics of incorporation of tritium into the purine sites of the polymers are identical with those for the corresponding purine mononucleotides. When the nucleic acids are in their native conformations, however, the purine labeling rates are reduced below that expected for the free mononucleotides. The magnitude of the effect is remarkably dependent upon the particular nucleic acid. For example, at 37 °C the purines in double-stranded DNA label at a rate two- to threefold slower than the corresponding mononucleotides, but in a double-stranded viral RNA, a 30- to 40-fold effect is found. The data suggest a strong influence of microscopic helix structure on the rate of tritium incorporation. First-order rate constants for the exchange of tritium into *specific* purine sites in yeast $tRNA^{Phe}$ were also determined. This was done by partially labeling the nucleic acid in tritiated water, and subsequently removing free and loosely bound tritium. Under conditions where exchange-out does not occur, the nucleic acid was digested with specific nucleases; chromatographic separation then enabled specific activities of purines from specific sites to be obtained. The rate constants for these sites show a large variation. They are markedly reduced for those residues occurring in cloverleaf helical sections and, in certain cases, for those known from crystallographic data to be involved in tertiary interactions. As examples of bases that can participate in tertiary interactions, the crystal structures show A14 and G15 in special base-pairing arrangements. Both purines (A14 and G15) occur in single-stranded sections of the cloverleaf; both show markedly reduced C-8 hydrogen-exchange rates. On the other hand, rate constants for bases and regions known to be on the outside of the molecule—such as the anticodon loop and the 3′ terminus—are perturbed the least. In one instance, a base in the dihydrouridine loop believed to be involved in tertiary interactions, according to crystallographic studies, incorporates tritium as if it were relatively unperturbed by the tRNA structure. The structural interactions of this base may be partially or completely broken at 37 °C in solution.

A wide variety of approaches have been used to study nucleic acid structure and conformation in solution. Although these approaches have yielded a diverse body of important information, no single technique has provided a picture of the local structure and environment at sites distributed throughout the entire molecule. Optical spectra, for example, generally give a superposition of overlapping bands arising from residues in diverse sections. Likewise, although discrimination is better with NMR, sites with similar resonance characteristics are visualized collectively in a typical spectrum. Site specific probes and chemical modifications are generally applicable to only a few select positions, and always possess the inherent danger that their introduction significantly perturbs the structure.

These shortcomings and difficulties are significantly overcome in a tritium labeling approach which takes advantage of the slow exchange of the C-8 hydrogen of purine residues (Gamble and Schimmel, 1974). Since the exchange is a pseudo-first-order process (Maeda et al., 1971; Tomasz et al., 1972; Elvidge et al., 1971, 1973, 1974; Livramento and Thomas, 1974), the incorporation of ^3H into purine P_i located at site i is given by

$$-\frac{d\Delta\hat{P}_i}{dt} = \frac{\Delta\hat{P}_i}{\tau_i} \quad (1)$$

where $\Delta\hat{P}_i$ is the deviation in the concentration of tritium labeled purine $\hat{P}_i$ from its final value achieved at isotopic equilibrium and τ_i is the time constant associated with exchange at site i. The approach to isotopic equilibrium for the purine at site i is, therefore, a simple exponential process given by:

$$\Delta\hat{P}_i = \Delta\hat{P}_{i0}e^{-t/\tau_i} \quad (2)$$

where $\Delta\hat{P}_{i0}$ is the value of $\Delta\hat{P}_i$ at $t = 0$. In a nucleic acid of independently exchanging units, the rate of total purine labeling, $\Delta\hat{P}_t$, is a sum of exponentials

$$\Delta\hat{P}_t = \sum_i \Delta\hat{P}_i = \sum_i \Delta\hat{P}_{i0}e^{-t/\tau_i} \quad (3)$$

where the summation is over all purine sites in the macromolecule.

The time constant τ_i associated with exchange at site i depends significantly upon the microenvironment at that site (Maslova et al., 1969; Lesnik et al., 1973; Gamble and Schimmel, 1974). A measurement of τ_i, therefore, is an indicator of the local environment and of structural interactions of the purine at site i. The great advantage of the C-8 labeling approach is that time constants for exchange may be measured

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for individual sites distributed throughout the whole molecule (Gamble and Schimmel, 1974). This is accomplished by labeling the molecule until sufficient tritium has been incorporated into the various sites; the tritium in the solvent and in rapidly exchanging positions in the nucleic acid is then removed. The polymer is subsequently digested into small pieces by specific nucleases. These are separated and each is then further degraded in order to obtain specific A's and G's from individual fragments. All of these procedures are carried out under conditions where exchange-out from the C-8 position does not occur to an appreciable extent. Once labeled purines from defined sites have been obtained, incorporation of tritium (and τ_i) is readily determined by a simple specific activity measurement.

This approach is not to be confused with, and has a decidedly different molecular basis than, the powerful and useful hydrogen-exchange procedure developed by Englander (1963). In the latter case, relatively rapid exchanging hydrogens are monitored. These are generally from hydrogen-bonded loci in the molecule. Moreover, in this case only the total exchange ($\Delta\hat{P}_t$) is followed; it is unfeasible to determine the time constants associated with specific sites. In spite of this limitation, the approach has been extremely fruitful (e.g., see Printz and von Hippel, 1968; Englander et al., 1972a,b; Englander and von Hippel, 1972).

In the present paper, the rate of exchange at the C-8 position is examined for single-stranded poly(A), poly(A):poly(U), double-stranded viral RNA, tRNA, and DNA. Not only are rate differences found for ordered and disordered forms of a given polymer, but substantial differences exist between the various nucleic acids in their ordered forms. In particular, the exchange rates show a remarkable variation among the helical polymers. All of these data provide a reference frame into which results on a polymer of unknown structure may be projected. In the case of yeast $tRNA^{Phe}$, rate constants for exchange have been obtained for specific sites within the native molecule. These are compared with expected behavior based on results with the other polymers, and on the microenvironments of the bases as seen in the crystal structure (Kim et al., 1974a; Robertus et al., 1974).

Materials and Methods

Materials. Mononucleotides and nucleosides used as standards in various experiments were purchased from Sigma. Poly(A), poly(U), and poly(A):poly(U) duplex polymers of mol wt > 100 000 were obtained from Miles and used without further purification. In some experiments the poly(A):poly(U) duplex was made from poly(A) (type I) and poly(U) (type II) obtained from Sigma. In these cases the duplex was generated by high-temperature annealing of the two polymers. Results with the duplexes from different sources were similar. Calf thymus DNA was also purchased from Sigma. The RNA duplex from *Penicillium chrysogenum* virus was a generous gift of Dr. Hugh D. Robertson, Rockefeller University.

Yeast $tRNA^{Phe}$ was purchased from Boehinger Mannheim or purified from unfractioned tRNA (Boehinger Mannheim) by the method of Litt (1968). It was devoid of significant contaminating tRNAs or defects, according to amino acid acceptance and T1 fingerprint analyses carried out in this laboratory. Concentrations were determined by assuming a specific activity of 1830 pmol/(A_{260} at around neutral pH) (Wimmer et al., 1968).

Sankyo brand T1 and T2 RNases were purchased from Calbiochem. Units are defined by Takahashi (1961) and Uchida (1966), respectively. DNase I and bacterial alkaline phosphatase (BAPF)[1] were obtained from Worthington; units of DNase I activity are given by Kunitz (1950).

Thin-layer chromatography was carried out on 20 × 20 cm glass plates coated with 0.1 mm of cellulose (Celplate-22, Brinkman). Compositions by volume of the various solvents used were: solvent I—55% 1-propanol, 35% H_2O, and 10% concentrated NH_4OH; solvent II—66% isobutyric acid, 33% H_2O, and 1% concentrated NH_4OH; solvent III—80% 1-propanol, 10% H_2O, and 10% concentrated NH_4OH.

Tritiated water (5 Ci/ml) was purchased from New England Nuclear. Because of the high specific activity, the M.I.T. High Intensity Radio Isotope Storage Facility was used for storage and for conducting experiments. Standard precautions and regulations were observed.

Methods. In a typical experiment, the nucleic acid to be tritiated was lyophilized from a low salt buffer and then taken up in a sodium cacodylate buffer (pH 6.5) containing the desired concentrations of salt, Mg^{2+}, and EDTA. Tritiated water was then added to give a final volume of 10–50 μl and a total nucleic acid concentration of 10–50 mg/ml. The plastic snap-top reaction vial was placed in a larger screw-cap plastic bottle and submerged upright in a water bath. Incubations varied from 72 h at 30 °C to 20 min at 100 °C, always keeping incorporation to less than 20% of maximum, in order to keep within the range where labeling is linear with time. The pH of the buffer was found to be temperature independent over a 40 °C range. Moreover, since the C-8 exchange is pH independent around pH 6.5 (Maeda et al., 1971; Tomasz et al., 1972; Elvidge et al., 1973, 1974), small variations in pH are inconsequential.

Following incubation, the sample was lyophilized to 6–8 times with the residue formed at the end of each lyophilization taken up in 50–100 μl of H_2O. After these lyophilizations, the radioactivity in the sample remained unchanged with further lyophilizations. An alternate procedure sometimes used involved terminating the reaction with 0.3–0.5 ml of a DEAE-cellulose slurry in 0.02 M sodium cacodylate (pH 5.5). The DEAE-cellulose was then washed into a small Pasteur pipet plugged at the lower end with glass wool. The excess tritiated water and tritium in fast exchanging positions were removed by carefully washing the column with about 5 ml of the equilibrating buffer. Bound material was eluted with 1 M NaCl or 1 M NH_4OAc. When nuclease digestions were to be carried out, the NH_4OAc eluant was lyophilized.

T1 RNase digestions of $tRNA^{Phe}$ were carried out (total volume of 100 μl) with 10 A_{260} units of $tRNA^{Phe}$, 60–100 units of T1 RNase, and 20–30 μg of BAPF in 10 mM NH_4HCO_3, pH 7.5. Digestion proceeded for 4–5 h at 37 °C, and was followed by lyophilization. The residue was taken up in 5–10 μl of H_2O, and approximately 1.5 A_{260} units were spotted on each cellulose thin-layer plate. Chromatography was done first with solvent I and then with solvent II (at a right angle). The separated oligonucleotides were visualized under uv light; their identities were established by standard procedures (G. P. Budzik, in preparation; see also Budzik et al., 1975). Fragments 7A and 10A (see below) cochromatographed in this

[1] Abbreviations used are: BAP, bacterial alkaline phosphatase; m^1A, 1-methyladenosine; m^7G, 7-methylguanosine; Gm, 2′-*O*-methylguanosine; m^2G, N^2-methylguanosine; m_2^2G, N^2-dimethylguanosine; EDTA, (ethylenedinitrilo)tetraacetic acid; P_i, inorganic phosphate; TLC, thin-layer chromatography; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NMR, nuclear magnetic resonance; AMP, adenosine 5′ monophosphate; GMP, guanosine monophosphate.

system and after elution were separated by electrophoresis (Jekowsky, 1975).[2]

Oligonucleotides were obtained by scraping the spots from the plates and suspending the cellulose in ca. 1 ml of 0.01 M NH_4OAc, pH 4.5. The cellulose was removed by centrifugation. Concentrations were determined by optical density measurements, assuming that the extinction coefficient of an oligonucleotide is simply the sum of the extinctions of each of the constituent bases, with an appropriate hypochromicity correction. Published values of extinction coefficients were used and hypochromicity corrections were obtained by digesting each oligonucleotide with T2 RNase and measuring the subsequent change in optical density (see Gamble, 1975). Radioactivities were determined by suspending the samples in 3-6 ml of Aquasol (New England Nuclear) and counting by scintillation. The error in the specific activity measurements is estimated as within ±10%.

In certain instances, tRNA or the T1 oligonucleotides were digested to mononucleotides with T2 RNase. The typical digestion mixture contained (in 100 μl) one A_{260} of nucleic acid and two units of T2 RNase in 50 mM NH_4OAc buffer, pH 4.5. Incubation was for 5 h at 37 °C and was followed by lyophilization. The nucleotides were converted to nucleosides by treatment (in 100 μl) with BAPF (3–5 $\mu g/A_{260}$ unit of nucleic acid) for 4–5 h at 37 °C in 10 mM NH_4HCO_3, pH 8.0. The nucleosides were lyophilized, separated by chromatography on thin-layer plates with solvent III, or in two dimensions with solvent I and solvent II, and subsequently visualized under a uv lamp.

Controls were carried out to check for loss of tritium during the TLC procedures. AMP and GMP were tritiated at 100 °C and isolated by successive lyophilizations or by the DEAE procedure described above. The specific activities were determined before and after running thin-layer chromatograms with solvents I, II, and III. Specific activities of the chromatographed nucleotides differed by less than 10% from those determined before chromatography. Thus, exchange-out from the purines is inconsequential in these solvents.

Under certain conditions, however, tritiated tRNA isolated by successive lyophilizations or the DEAE procedure is contaminated with radioactivity which is lost upon chromatography of its T1 fragments. This difficulty was overcome by spotting the freshly isolated [^{3}H]tRNA on a thin-layer plate and performing chromatography with solvent III. Although the tRNA remains stationary, the contaminating radioactivity is removed; the nucleic acid was then eluted and subjected to further analysis (e.g., T1 digestion). After such treatment, the radioactivity in the tRNA was constant; for example, the sum of the radioactivities of the T1 fragments isolated by chromatography in solvents I and II equaled that of the intact tRNA before T1 digestion. The origin of the contaminating radioactivity is unknown, but its contribution to the total activity was greater at lower incubation temperatures, e.g., 37 °C. More details are given by Gamble (1975).

In some experiments, [^{3}H]DNA was chromatographed with solvent III before the specific activity was determined. The cellulose containing the tenaciously bound DNA was scraped and incubated (in 500 μl) overnight at 37 °C with DNase I (10 $\mu g/A_{260}$ unit of DNA) in 10 mM $MgCl_2$, 10 mM Tris buffer, pH 7.5. This procedure released the DNA from the cellulose. Centrifugation removed the cellulose, and the optical density and radioactivity of the deoxyoligonucleotides were measured as described above.

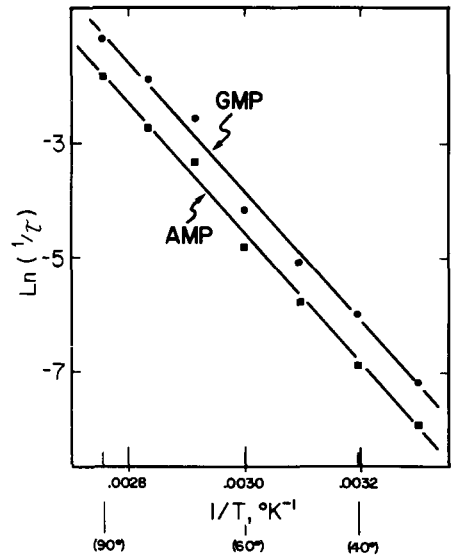


FIGURE 1: Ln $(1/\tau)$ vs. $1/T$ for the exchange of tritium into the C-8 position of AMP and of GMP. Solutions contained 50 mM sodium cacodylate (pH ≃ 6.5), 10 mM EDTA, and about 40 mM of the nucleotide. Units on $1/\tau$ are h^{-1}.

Results

Labeling of AMP and GMP. The tritium labeling rates of the mononucleotides AMP and GMP are important references for the labeling of the corresponding nucleotide units in polymers (see below). Rate constants (reciprocal time constants, τ) were determined by carrying out exchange for times short compared to τ so that the exponential in eq 2 may be expanded to give

$$\frac{\Delta\hat{P}}{\Delta\hat{P}_0} = 1 - t/\tau, t \ll \tau \qquad (4)$$

where P is AMP or GMP. Rate constants thus determined at a series of temperatures between 30 and 90 °C are given in Figure 1, which plots log $(1/\tau)$ vs. $(1/T)$. These parameters were obtained at pH 6.5, which is well outside the region where the exchange is pH dependent (Maeda et al., 1971; Tomasz et al., 1972; Elvidge et al., 1973, 1974). It is clear from Figure 1 that at all temperatures GMP exchanges about two-fold more rapidly than AMP. This agrees with the results at 37 °C of Tomasz et al. (1972), of Elvidge et al. (1973, 1974) at 85 °C, and of Shelton and Clark (1967) at 100 °C. Over the range of 35–80 °C, the rate constants for AMP are within <40% of those determined by Maslova et al. (1969) for the same exchange reaction (^{1}H-8 → ^{3}H-8). The exchange rates are extremely temperature dependent, giving activation energies in slight excess of 22 kcal/mole^{-1} for both nucleotides. This value is within the range of activation energies reported for adenine, adenosine, and AMP (Maslova et al., 1969; Maeda et al., 1971; Elvidge et al., 1971, 1973; Livramento and Thomas, 1974).

Because Mg^{2+} was used to stabilize nucleic acid structures in experiments reported below, the effect of Mg^{2+} on the rate of labeling of AMP and GMP was investigated. At pH 6.5, the presence of 10 mM Mg^{2+} did not significantly affect the rate of ^{3}H incorporation into either nucleotide.

Labeling of Nucleic Acid Polymers. The labeling of A's and G's in several nucleic acid polymers was studied under condi-

[2] In an earlier paper (Gamble and Schimmel, 1974). Fragment 7A was believed to cochromatograph with 7B. This error was later discovered and unequivocal results were obtained by electrophoretic separation of 7A from 10A. Also, the *R* values for the various fragments need some adjustment in Gamble and Schimmel (1974), due to a normalization error.

tions where ordered, disordered, and mixtures of conformations were present. In treating such data, it is convenient to express labeling rates in relative terms. This is accomplished as follows. Recognizing that $\Delta\hat{P}_{i0}$ in eq 3 is the same for all purines ($=\Delta\hat{P}_0$), and that at sufficiently short times t the exponential may be expanded to first order in t, we obtain

$$\frac{\Delta\hat{P}_t}{N\Delta\hat{P}_0} = 1 - \frac{t}{\bar{\tau}}, \; t \ll \bar{\tau} \tag{5}$$

where

$$\frac{1}{\bar{\tau}} = \frac{1}{N}\sum_i \left(\frac{1}{\tau_i}\right) \tag{6}$$

and N is the total number of purines in the polymer. The left-hand side of eq 5 is the fractional deviation of the exchange of the whole molecule from its final equilibrium value, and $\bar{\tau}$ is an average time constant. This equation is exactly analogous to that for a single site (eq 4).

The theoretical average time constant $\bar{\tau}_0$ for a polymer in which the A and G residues behave as open units exchanging like free AMP and GMP residues is given by

$$\frac{1}{\bar{\tau}_0} = \frac{f_A}{\tau_A{}^0} + \frac{f_G}{\tau_G{}^0} \tag{7}$$

where f_A and f_G are the fraction of A and of G residues, respectively; the parameters $\tau_A{}^0$ and $\tau_G{}^0$ are time constants for labeling of free AMP and GMP, and are given for any given temperature in Figure 1.[3] Therefore, the experimentally determined parameter is $\bar{\tau}$ (eq 5 and 6), while $\bar{\tau}_0$ may be calculated from the base composition and $\tau_A{}^0$ and $\tau_G{}^0$ values (eq 7).

The parameter R is a dimensionless ratio defined by:

$$R = \frac{\bar{\tau}}{\bar{\tau}_0} \tag{8}$$

Thus, R is a measure of the time constant for labeling relative to that for a mixture of free AMP and GMP present in the same proportions as in the polymer. It is convenient to use this parameter in the presentation of experimental data. R values reported in the figures and table are typically accurate to ±15%.

Some results are tabulated in Table I for five different polymers—poly(A), poly(A):poly(U), DNA, tRNA, and double-stranded RNA from *Penicillium chrysogenum* virus. Various temperatures were employed in order to give different states of helicity and folding. In all cases examined, the polymers are completely melted out at the highest temperature employed, according to hypochromicity measurements. The R values for the different melted polymers are each within experimental error of 1.0. This means that incorporation of AMP or GMP into a random nucleic acid polymer, regardless of the composition of the polymer, does not perturb labeling at the C-8 position.

On the other hand, substantial departures of R from unity occur at temperatures where ordered or partially ordered structures are formed. Poly(A), for example, assumes a value of R = 1.6 at 60 °C where it is about 30–35% stacked and R = 3.1 at 37 °C (55–65% stacked; Leng and Felsenfeld, 1966; Brahms et al., 1966). As Maslova et al. (1969) have also noted, these results show that stacking of the bases produces a retardation in the rate of isotope exchange at C-8.

The behavior of poly(A):poly(U) is particularly interesting, however. Under the conditions of the experiments, it undergoes a sharp melting transition centered at 67 °C. At 37 and at 60 °C there is little change in hypochromicity, indicating the duplex structure is almost completely intact at both temperatures. Nevertheless, there is a large difference in relative labeling rates at these two temperatures (R = 4.8 at 60 °C and R = 17 at 37 °C). This difference implies that the apparent activation energy for labeling the poly(A):poly(U) duplex is greater than that for AMP. This in turn might be due to sensitivity of the H-8 exchange to the frequency of helix fluctuations (e.g., breathing modes (Utiyama and Doty, 1971; Englander et al., 1972b)), or to subtle differences in the helix parameters at the two temperatures.

The most striking result in Table I is the sizable differences between the three duplexes at 37 °C. The exchange rates of purines in DNA are only two- to threefold less than those of free mononucleotides; at the other extreme, purines in the natural double-stranded RNA exchange at rates 30- to 40-fold less than those of free mononucleotides. The exchange rate for the poly(A):poly(U) duplex at 37 °C assumes an intermediate value. The results on DNA are in approximate agreement with those of Tomasz et al. (1972) and Iida et al. (1974). The large difference between helical DNA and the helical RNAs could arise from special effects created by the 2′-OH in the duplex of the RNA polymers. But since differences exist between the RNA polymers, the H-8 exchange could also be sensitive to the microscopic helix structure, apart from any chemical effects of the 2′-OH. In this connection, it is interesting to note that the microscopic environment of bases in a duplex is very

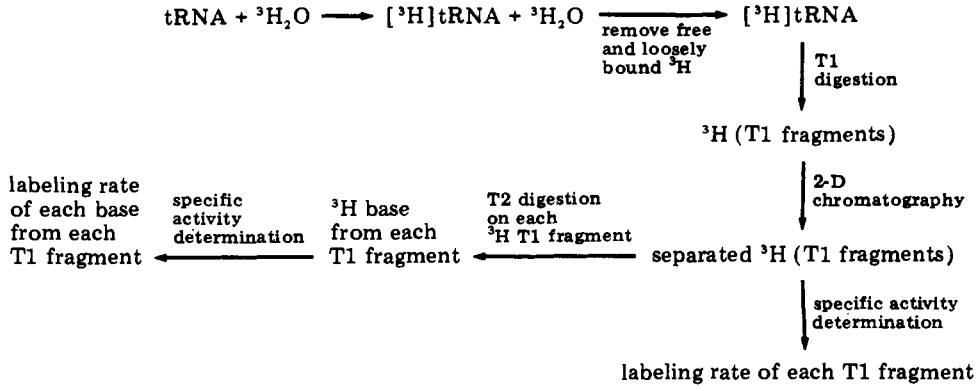
TABLE I: C-8 Tritium Labeling Rates for Various Nucleic Acids: Values of R[a].

Poly(A)	Poly(A):Poly-(U)	Viral RNA	tRNA	DNA[e]	Temperature (°C)
1.05	0.97		1.0[b]	0.97[c]	90–100
1.6	4.8		4.5	1.6	60
3.1	17	36(A)[d] 44(G)[d]	7.9	2.6	37

[a] All incubations (except as noted) contained ca. 50 mM sodium cacodylate (pH ≃ 6.5), 1 mM EDTA, 10 mM Mg^{2+}, and variable additional monovalent cation. Except for DNA and tRNA, purines were separated from pyrimidines in the specific activity determination so that true purine R values are given. In the case of tRNA and DNA, a correction for the pyrimidine labeling contamination is estimated to increase the R values by less than 20% at 37 °C and by less than 10% at 60 °C, and 90–100 °C. [b] Incubation mixture contained ca. 50 mM sodium cacodylate (pH ≃ 6.5), 10 mM EDTA, and no Mg^{2+}. [c] Incubation mixture contained ca. 50 mM sodium cacodylate (pH ≃ 6.5), 10 mM EDTA and no Mg^{2+}. [d] T2 digestions were performed to give separate values for the A's and G's. [e] In calculating R values for DNA, we have used τ^0 values for the ribonucleotides. This appears to be valid since Maeda et al. (1971) showed that under their conditions exchange rates were closely similar for adenosine, 2′-deoxyadenosine, and 2′,3′-isopropylideneadenosine. Also, using the ribonucleotide τ^0 values, R = 1 for melted DNA, as expected.

[3] Values of τ_0 for the various modified bases are not known for the conditions of the experiments done here. We assume that τ^0 for Gm (fragment 13) is the same as that for G. It is reasonable to assume that modifications of the ribose group, such as in Gm, have no significant effect on τ^0, especially since exchanges at C-8 for adenosine, 2′-deoxyadenosine, and 2′,3′-isopropylideneadenosine have almost the same pseudo-first-order rate constants (conditions different than those employed here; Maeda et al., 1971). For the purpose of constructing Figure 3 bases modified on the pyrimidine moiety of the purine ring were also assumed to have τ^0 values identical with their unmodified counterparts. An exception is $m_2{}^2G$ for which $\tau^0{}_{m_2{}^2G}$ was separately measured. In the case of fragment 12 in Figure 3, only the R value for the unmodified G is given. Figure 4 tabulates only bases for which accurate τ^0 values are known (i.e., A, G, and $m_2{}^2G$).

Scheme I: Scheme for Determining Labeling Rates of Individual Bases in Transfer RNA.



sensitive to helix parameters (Arnott et al., 1969). In addition, due to the lack of G:C pairs breathing reactions in the poly(A):poly(U) duplex may be more prevalent than in the natural RNA helix; this in turn may give more rapid H-8 exchange for poly(A):poly(U) compared to the viral RNA. Finally, neighboring bases may exercise a chemical influence on a given base in a duplex; certainly the A's in the viral RNA duplex have a wide variety of neighbors compared to those in poly(A):poly(U).

The R value of tRNA^{Phe} (T_m = 77 °C) is much larger at 37 than at 60 °C where the polymer is melted to a disproportionately small degree (ca. 20%). This may be due to the nonlinear relation between fractional melting and R (see below) and, additionally or alternatively, to temperature-dependent effects that alter the rate of H-8 exchange in tRNA just as they do in poly(A):poly(U). Finally, the R value of tRNA at 37 °C is intermediate between that of poly(A) and those of the RNA duplexes, a finding consistent with the mixture of single- and double-stranded regions found in the tRNA structure.

Labeling at Specific Sites in tRNA: T1 Fragments. A detailed examination of labeling at specific sites in tRNA^{Phe} was undertaken. The sequence and cloverleaf structure of this tRNA are given in Figure 2. The figure also denotes the fragments produced by digestion with T1 ribonuclease. The fragments are numbered in accordance with their positions on a two dimensional chromatogram.

The general procedure used for studying discrete sites in tRNA is outlined in Scheme I. As shown by this scheme, labeling at discrete sites was approached at two levels of resolution. In the first of these, the nucleic acid was digested with T1 RNase, after incubation in TOH. The individual T1 fragments were separated and specific activities were determined as described in Materials and Methods. R values for each fragment were then calculated by eq 8. The next higher stage of resolution was achieved by degrading each of the separated T1 fragments to the mononucleotide level with T2 RNase. The lone G and the A's (if any) were then separated and the individual purine specific activities were determined. In this way the time constant τ_i (or R value) for a specific nucleotide in the molecule is obtained, except for cases in which either multiple A's occur in a T1 fragment or the bases themselves arise from redundant T1 fragments.

Figure 3 gives results obtained for yeast tRNA^{Phe} at 37 °C (10 mM Mg^{2+}) at the resolution level of the T1 fragments. The figure gives R values in bar graph form for the various fragments numbered in Figure 2. These show labeling rates that are markedly dependent on the positions of the fragments in the sequence. Some of these fragments are redundant, occurring more than once in the structure, while others come from unique loci and contain only a single purine (e.g., fragments 4, 5, 8, and 9). In all cases, the time constants for exchange are longer than what is predicted for free AMP and GMP residues. In some instances, the exchange is retarded by 20-fold or so (e.g., fragments 4, 7a, 10a, and 10b); in others, the retardation is only three- to four-fold (e.g., fragments 6 and 8). Therefore, the profile of labeling rates given in Figure 3 is potentially a rich source of interesting structural information.

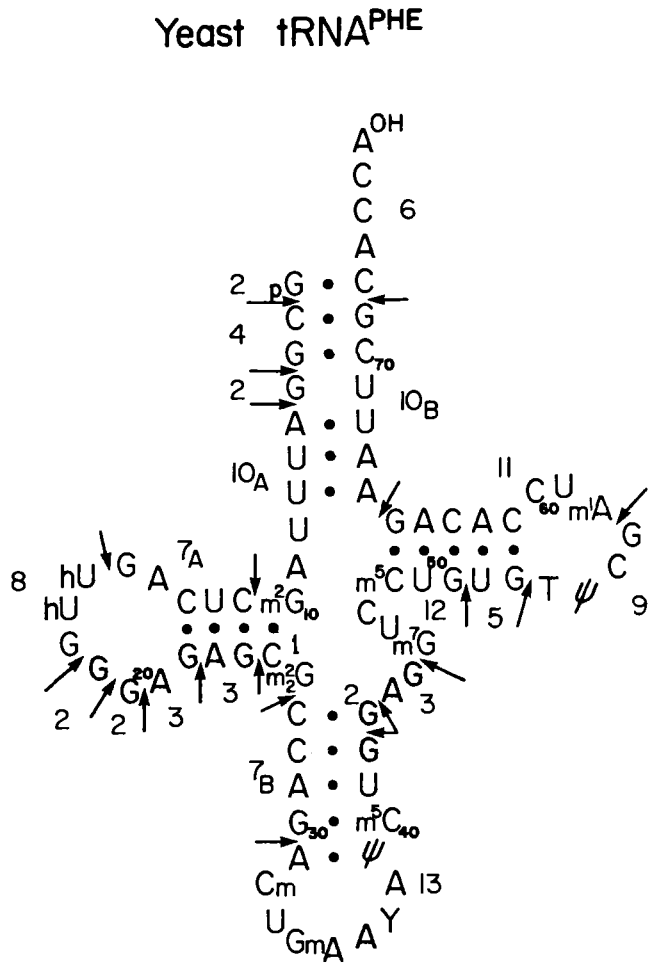


FIGURE 2: Sequence and cloverleaf structure of yeast tRNA^{Phe} (RajBhandary and Chang, 1968). The T1 fragments are indicated and numbered in accordance with their positions on a two-dimensional chromatogram. The numerical position of every tenth base in the sequence is designated by a lower case number.

Earlier studies at 90 °C in the presence of EDTA showed that R values for fragments derived from the unfolded molecule fluctuate around unity (Gamble and Schimmel, 1974).

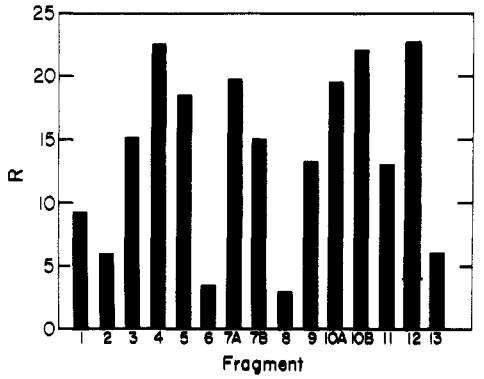


FIGURE 3: R values for fragments derived from intact yeast $tRNA^{Phe}$ that was incubated in TOH at 37 °C in 50 mM sodium cacodylate (pH 6.5) and 10 mM Mg^{2+}. The tRNA concentration was 12.5 mg/ml.[3]

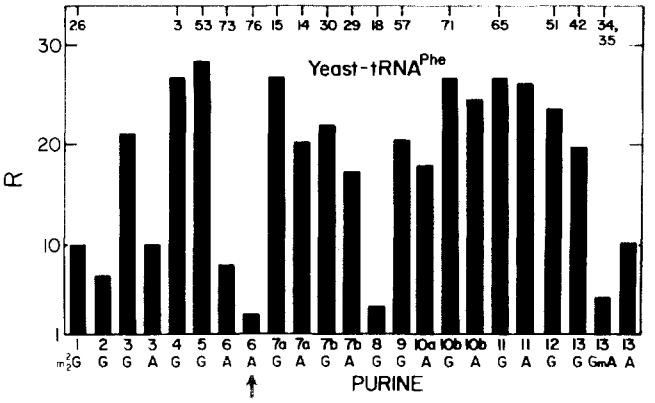


FIGURE 4: R values for individual bases in yeast $tRNA^{Phe}$ that was incubated in TOH at 37 °C in 50 mM sodium cacodylate (pH 6.5) and 10 mM Mg^{2+}. The tRNA concentration was 12.5 mg/ml. The abscissa gives the base (A or G) from the numbered T1 fragments (see Figure 2). The numbered tick marks across the top of the figure identify the numerical positions of bases in the sequence. GmA denotes 2′-*O*-methyl GpA[3]. An arrow is placed beneath the 3′-terminal A.

Other experiments at 37 °C with free T1 oligonucleotides of *E. coli* $tRNA_2^{Tyr}$ showed that $R \simeq 1$–2 for the purines in the oligomers, the small excess of R over unity presumably being due to stacking effects in the fragments (Schoemaker, 1975). Collectively these results, like those in Table I, established that the labeling rates of bases in disordered polymers are similar to those for free AMP and GMP.

The purine exchange rates in Figure 3 contain an error due to pyrimidine labeling that was not accounted for in the calculation of $\bar{\tau}_0$. The labeling of the tRNA pyrimidines, which presumably occurs at C-5 (Fink, 1964; Wataya et al., 1972; Iida et al., 1974), is small (<5%) relative to purine labeling at 90 °C, but at 37 °C the pyrimidines account for 10% or more of the labeling of some fragments, depending on their pyrimidine content (Gamble, 1975). This error is corrected by performing T2 digests on each of the T1 fragments and subsequently separating purines from pyrimidines (see below).

The incubation mixture for the experiments summarized in Figure 3 contained 12.5 mg/ml of $tRNA^{Phe}$. Experiments at 25 mg/ml gave similar results. A more extensive concentration range was investigated in the case of *E. coli* $tRNA_2^{Tyr}$. In this case, R values for individual T1 fragments were the same (within experimental error) for incubations at 2.5 mg/ml, 10 mg/ml, and 50 mg/ml (Schoemaker et al., 1976). Thus, at the concentrations employed, the retarded exchange rates of bases within tRNA have little contribution from potential aggregation effects.

The experiments cited in Figure 3 can be criticized on the grounds that the observed effects could result from a direct influence of Mg^{2+} on the C-8 exchange. Although results cited earlier showed that 10 mM Mg^{2+} does not perturb the labeling of AMP or GMP, it can be argued that the strong interactions of Mg^{2+} at specific loci on the folded RNA structure (cf. Schreier and Schimmel, 1974) are entirely different in character than the relatively weak Mg^{2+}–mononucleotide association (Smith and Alberty, 1956). To check on this point, tritium labeling was carried out with 10 mM spermine, an oligovalent polyamine cation known to be effective in stabilizing nucleic acid structure (Hurwitz and Rosano, 1967; Liquori et al., 1967; Sulwalsky et al., 1969; Leboy, 1970; Igarashi and Takeda, 1970; Robison and Zimmerman, 1971; Cohen, 1971; Schreier and Schimmel, 1975). The results obtained were very similar to those shown in Figure 3. Therefore, Mg^{2+} has little direct influence on the H-8 exchange in tRNA, and the large variation in the labeling rates of specific sites is due to true structural effects.

Labeling at Specific Sites in $tRNA^{Phe}$: Specific Purines. In order to obtain more precise information, and to eliminate ambiguities arising from the contaminating radioactivity of pyrimidines, the T1 fragments derived from the tritiated $tRNA^{Phe}$ were subjected to T2 RNase digestion and the resulting monomer units were separated and analyzed. The results obtained for yeast $tRNA^{Phe}$ are depicted in Figure 4, which gives R values for many different sites. For each position on the abscissa, the number of the T1 fragment is given together with the base (A or G) from that fragment. In cases where unique bases can be specified, the tick marks and numbering across the top of the figure indicate their positions in the sequence.

The specific activities of the pyrimidines from the various fragments were also determined. When these were combined with the data of Figure 4, specific activities for individual T1 fragments could be calculated from the constituent mononucleotide activities. These calculated activities are generally within 20% of the values given by the original determinations on the intact T1 fragments (Figure 3).

Figure 4 contains a great deal of interesting structural information. Consider first those purines which are derived solely from cloverleaf helical sections. These include purines from fragments 4, 5, 7b, 10b, and 11, as well as the guanosines from 10a (m^2G), 12, 13. Collectively they represent 12 purines scattered throughout different helical sections of the cloverleaf. The R values for these bases fall in the range of ca. 17–29. Since in every case the labeling is retarded 17-fold or more, the environment of the cloverleaf helix clearly has a strong influence on C-8 labeling; the large R values for the cloverleaf bases also suggest that at 37 °C no significant breaks in the helices occur at any of these loci.

On the other hand, there are sections that are significantly less perturbed by the tRNA structure. These include, for example, the A's of fragment 6 and the 2′-*O*-methyl GpA (GmA) of fragment 13. (The dinucleotide is produced because T2 RNase does not cut after a 2′-*O*-methyl group.) Available solution and x-ray data indicate that these parts of the molecule are not involved in special intramolecular interactions and appear on the "outside" of the structure (Brostoff and Ingram, 1967; Uhlenbeck, et al., 1970; Cashmore et al., 1971; Pongs et al., 1970; Uhlenbeck, 1972; Cameron and Uhlenbeck, 1973; Kim et al., 1973, 1974a; Robertus et al., 1974). The relatively small retardation in labeling in these areas is probably due to

base stacking interactions (see below).

In terms of R, the most rapidly exchanging site is A76. This base, which occurs in fragment 6, is designated by an arrow in Figure 4, while the other A in this fragment is A73. (A76 was separated from A73 by digesting whole $tRNA^{Phe}$ in the absence of BAP. Subsequent chromatography gave separation of Ap from A_{OH}(A76). The R value determined for A76 was then used in conjunction with the R value for the two A's of fragment 6 to calculate R for A73.) Exchange at A73 is about three times slower than at A76, even though both are in the same single-stranded section of the tRNA structure. This difference may be attributed to the stacking interaction of the internal A (A73) with the tail end of the acceptor helix. By being further removed from the end of the helical duplex, the terminal A is less likely to be constrained by its influence. It is also noteworthy that the exchange rate at the presumably stacked A73 is quite comparable to anticodon bases G34–A35, which are stacked to some degree in the crystal form (cf. Kim et al., 1974b).

The most interesting question, however, is whether the diverse tertiary interactions visualized in the high-resolution crystal structure (Kim et al., 1974a; Robertus et al., 1974) are also reflected in the labeling pattern of the molecule in solution. Many of these tertiary interactions involve bases within single-stranded portions of the cloverleaf, and presumably are common to all tRNAs.

Consider first the TΨC loop. The guanine of G57, the lone purine of fragment 9, interacts with the dihydrouridine loop in the crystal structure. As can be seen in Figure 4, the labeling of this single-stranded base is markedly retarded ($R \simeq 20$), which clearly points to a major perturbation of its environment.

On the other hand, the x-ray data also specify an interaction of G18 and G19 of the dihydrouridine loop with the TΨC loop (Kim et al., 1974a; Robertus et al., 1974). Although the high redundancy of fragment 2 makes it difficult to draw conclusions on G19, the other base (G18) appears as the lone purine of fragment 8. The labeling of this base is relatively unperturbed ($R = 3.7$), however (Figure 4). It appears, therefore, that under the conditions of the experiment this base is not strongly interacting with another part of the tRNA structure. Other data obtained in solution also indicate that this base is quite accessible to the solvent (Pongs et al., 1971; Samuelson and Keller, 1972; Cameron and Uhlenbeck, 1973).

Another important tertiary interaction is the bonding between G15 and C48 (Levitt, 1969; Cashmore, 1971; Kim et al., 1974a; Robertus et al., 1974). Exchange at G15 is among the most retarded in the molecule, with a R value over 25. This suggests a strong structural interaction in solution for this base.

A special feature of the $tRNA^{Phe}$ crystal structure is the three regions that each contain hydrogen-bonded triples (Kim et al., 1974a; Robertus et al., 1974; Klug et al., 1974). These triples are C13:G22:m^7G46, U12:A23:A9, and U8:A14:A21. In the first two, a base from a cloverleaf single-stranded region (m^7G46 and A9) is paired with a base (m^7G46 with G22 and A9 with A23) that is already matched to its Watson–Crick complement in the cloverleaf helix. In the third, each is in a cloverleaf single-stranded section and the three bases bond by a unique scheme, which presumably involves both the base and ribose moiety of U8 (Kim et al., 1974b). Since G22 and A23 have retarded H-8 exchange rates by virtue of participation in the cloverleaf helix, their involvement in additional structural interactions cannot be appraised. But each of the remaining four purines (m^7G46, A9, A14, and A21) occur in single-stranded sections of the cloverleaf; perturbations in the exchange rates of these bases must be due to special features of the three dimensional structure.

The m^7G46 cannot be appraised, however, since the H-8 exchange of m^7G is presumably very rapid (Tomasz, 1970); exchange-out occurs during isolation of the tritiated tRNA.

In the case of A14, $R \simeq 20$. The threefold redundancy of fragment 3 and the lack of information on the contribution to the exchange rate of the A in fragment 3 that is from the extra loop make it unfeasible to estimate the exchange rate of A21. A9 occurs in fragment 10a. Since A5 is in a cloverleaf helical section, its R value must be *ca.* 17–29 ($R \simeq 24$ for the helical A's in fragment 10b). From this we can estimate the corresponding R value for A9 by writing (cf. eq 6 and 7)

$$\frac{\tau_A^0}{\bar{\tau}} = \frac{1}{2}\left[\frac{\tau_A^0}{\tau_{A5}} + \frac{\tau_A^0}{\tau_{A9}}\right] \tag{9}$$

where $(\tau_A^0/\bar{\tau})$ is the reciprocal of the observed R value and (τ_A^0/τ_{A5}) and (τ_A^0/τ_{A9}) are the reciprocal R values for A5 and A9. With R = 17–29 for A5, that for A9 is estimated from eq 9 as ca. 13–19. Although the nature of the perturbing interactions cannot be determined from the exchange kinetics data, evidently A14 and A9 are involved in additional structure not apparent from the cloverleaf.

Discussion

The results presented above establish that the rate of isotope exchange at the C-8 position of a purine base in a nucleic acid polymer is sensitive to the local structure around that base. Insertion of purines into a polymer per se does not alter the kinetics of exchange from that of free mononucleotides. This follows from the high temperature data in Table I, which show that random coil polymers of different base compositions incorporate tritium at the rate expected for the sum of their constituent mononucleotides. Moreover, Mg^{2+} per se does not affect the labeling rate, possibly because it is largely interacting with the backbone and not the bases per se. These observations are critical, since they enable us to conclude that deviations in labeling rates from those of the free mononucleotides must be due to effects of organized structure.

There is a large variation in the kinetics for the different double-stranded nucleic acid helices (see Table I). For double-stranded DNA at 37 °C, for example, the pseudo-first-order rate constants for the purine mixture in the polymer is only two- to threefold lower than that for the corresponding mixture of free mononucleotides; in the double-stranded viral RNA polymer, 30- to 40-fold effects are found. As mentioned earlier, these variations may reflect microscopic differences in helix structure (see Arnott et al., 1969), as well as other effects.

These observations, while interesting, bring out a weakness in the approach: there is no rigorous theoretical framework that correlates the first-order rate constant for exchange with microscopic structure. Such correlations are difficult to obtain because the effects are simply not large enough to give an unambiguous structural explanation. For example, a tenfold change in the rate constant for exchange corresponds to an activation energy change of only 1.5 kcal mol^{-1} out of a total of about 22 kcal mol^{-1}. Furthermore, it is possible that a rate constant may be perturbed to the same degree by two very different kinds of interactions. The data with tRNA show that remarkable reductions in the rate constants occur for bases in cloverleaf helical sections, as well as for certain ones involved in tertiary interactions.

Available data indicate the hydrogen exchange at C-8 proceeds through an ylide intermediate (Tomasz et al., 1972; Elvidge et al., 1973, 1974). The ylide is generated by proton-

ation at N-7 followed by proton extraction from C-8. The reaction cycle is completed by reprotonation at C-8 followed by hydrogen ion dissociation from N-7. With this mechanism, there are several ways in which the local nucleic acid structure can alter the exchange kinetics at a particular purine within a nucleic acid. One is that the local structure may alter the acidity ($pK \sim 18$; Elvidge et al., 1971) of the C-8 hydrogen. Local structure may also sterically affect the accessibility of solvent molecules to the C-8 position of a particular purine. But it is difficult to say which of these and other possibilities is the prime factor in the cases discussed above.

Although difficulties exist in establishing a molecular interpretation, the isotope labeling approach has considerable value when cast into a semiempirical framework. For example, data given above (e.g., with denatured polymers) show that residues free of structural interactions behave like simple mononucleotide units. For this reason, the approach is particularly useful for identifying those units which participate in structure formation, and those which do not. In addition, the data in Table I clearly show that the helices of DNA, synthetic RNA, and natural RNA have microscopic differences. At 37 °C, the exchange rates of the purines in these various helices vary over tenfold. No other solution technique can claim this kind of sensitivity to helix structure. The results for these three helices establish an empirical ruler. On this ruler, the cloverleaf helical sections of tRNA, which have R values of ca. 17–29, are more akin to the poly(A):poly(U) and natural viral RNA helices than to the DNA helix. This agrees with the crystallographic evidence that the parameters of the cloverleaf helices are approximately in line with those expected for an RNA helix (Robertus et al., 1974; Kim et al., 1974b).

As mentioned above, two very different local structures might perturb the exchange rate at a particular purine to the same degree. This gives rise to ambiguity in interpretation. On the other hand, when investigating a nucleic acid of known sequence but unknown secondary and tertiary structure, such as 5S RNA, certain logical procedures can be employed. These can facilitate building a structural model from the exchange data. For example, if a number of purines close together in the sequence have R values in the vicinity of 20, it is reasonable to conclude that they are all involved in helix formation with a complementary segment elsewhere in the molecule. Large R values for isolated purines scattered throughout the molecule, however, are probably due to the participation of these bases in tertiary interactions.

In principle the H-8 labeling approach can also be used to compute the fractional bonding of specific loci. Assume that the base at site i can exist in two states—bonded or unbonded—where the nature of the bonding is unspecified. Assume also R_{bi} and R_{ui} are the R values associated with bonded and unbonded states at site i, respectively. Proceeding by analogy with the derivation of eq 7 and 9, we can easily show:

$$f_{bi} = \frac{\left(\frac{1}{R_{ui}} - \frac{1}{R_i}\right)}{\left(\frac{1}{R_{ui}} - \frac{1}{R_{bi}}\right)} \qquad (10)$$

where R_i is the observed R value at site i and f_{bi} is the fractional occurrence of the bonded state at site i. Thus, with established values for R_{bi} and R_{ui} (obtained from an appropriate calibration), a measured value of R_i can be used to calculate f_{bi}.

Equation 10 is useful for giving insight into the physical significance of differences between sites in their respective R values, or of changes in the R value at one particular site. Assume for illustration that $R_{ui} = 3$ and $R_{bi} = 25$. With $R_i = 3$, then $f_{bi} = 0$, but with a rise of less than 5% of the maximal possible change to $R_i = 4$, then $f_{bi} = 0.27$. Thus, in this situation, a minimal change in R_i reflects a substantial change in fractional bonding. On the other hand, in the same example a change from $R_i = 25$ to $R_i = 20$ arises from a mere 3.4% change in f_{bi}. Hence, the relationship between R and a fractional difference or change in bonding can be markedly nonlinear. Of course, differences in R values between sites (e.g., in a cloverleaf helix) may additionally or alternatively be due to variations in microscopic environments around each purine rather than simply to the degree of bonding. Certainly the large differences in R values at 37 °C for the DNA and RNA helices must arise from such microscopic effects.

The particular power of the approach lies in the ability to monitor exchange at precise loci throughout the molecule. In the studies with yeast $tRNA^{Phe}$ discussed above, this was accomplished by digesting the labeled nucleic acid with T1 RNase and subsequently separating the T1 fragments. These were in turn degraded to mononucleotides with T2 RNase. This procedure permitted specific activity measurements to be made on single purines from known positions in the structure. However, in a number of instances the T1 fragments are redundant or an A occurred in more than one location within a T1 fragment. Ambiguities caused by these effects can be circumvented by using a different combination of nucleases, e.g., RNase A (which cuts after pyrimidines) in conjunction with T1 and T2 RNases.

The labeling pattern of yeast $tRNA^{Phe}$ in solution at 37 °C is consistent with many aspects of the crystal structure studied by Kim et al. (1974a,b) and Robertus et al. (1974). For example, the labeling pattern is entirely in accord with the occurrence of intact cloverleaf helical sections, of the relatively open and free environment of bases at the 3′-terminus and in the anticodon loop, and of tertiary structural interactions for A14, G15, G57, and probably A9. On the other hand, G18, another base believed to be involved in tertiary interactions in the crystal (Kim et al., 1974a; Robertus et al., 1974), evidently is free of tertiary bonding, or only partially bonded, at 37 °C in solution. This result may indicate that the G18 interaction is delicate and easily disturbed. In fact, strong involvement of G18 in tertiary structure formation is implicated in labeling data with other specific tRNAs (Schoemaker et al., 1976). Therefore, subtle effects of sequence and of overall structural stability may play a role in determining the intactness of a tenuous tertiary interaction.

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