Studies of Yeast Phenylalanine-Accepting Transfer Ribonucleic Acid Backbone Structure in Solution by Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy †

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ABSTRACT: Approximately 17 diester phosphates from the backbone structure of yeast tRNA^{Phe} give rise to phosphorus resonances, which are resolved in its ^{31}P NMR spectrum. To localize these diester phosphates within the tRNA structure, ^{31}P NMR spectra of several chemically or enzymatically modified yeast tRNA^{Phe} species were recorded. To this end selective modifications were performed in the anticodon, the DHU, and the TΨC loop. Modifications, performed in different loop regions, give rise to perturbation of different characteristic ^{31}P resonances. The ^{31}P spectra were correlated with the corresponding ^{1}H NMR spectra of the ring N hydrogen-bonded protons and interpreted in view of the X-ray results obtained on yeast tRNA^{Phe}. It is concluded that the diester phosphate groups, which experience an unusual shift, can be accounted for in the X-ray structure in terms of hydrogen-bonded phosphate groups and diester phosphates with a diester geometry, deviating from the normal double-helical conformation.

During the last five years much effort has been spent in characterizing the proton magnetic resonance spectra of transfer RNAs. Most of these studies were specifically directed toward the interpretation of spectra from hydrogen-bonded protons and methyl groups. These studies have yielded structural and dynamical information on transfer RNAs in solution (Kearns, 1977; Hilbers, 1979; Robillard & Reid, 1979). So far the information contained in the ^{31}P spectra has not been explored, although recently the potential of ^{31}P NMR applied to tRNAs has been indicated by Guéron & Shulman (1975). It was found that the ^{31}P NMR spectra consist of a large, structured resonance coming from the majority of the diester phosphates present in tRNA; in the case of yeast tRNA^{Phe}, approximately 60 diester phosphates contribute to this large resonance. In addition, a number of resolved resonances, covering a spectral region of roughly 8 ppm, are found up- and downfield from the main resonance. One of the isolated resonances could be assigned to the 5′-terminal monoester phosphate on account of its titration behavior (Guéron & Shulman, 1975). From the temperature dependence of the spectra, it was concluded that the other isolated resonances most likely arise from special foldings in the sugar phosphate backbone. Such special foldings are expected to occur in the loop regions on the basis of recent X-ray studies of yeast tRNA^{Phe} (Jack et al., 1976; Sundaralingam et al., 1976; Kim & Sussman, 1976; Quigley & Rich, 1976; Stout et al., 1978; Hingerty et al., 1978; Holbrook et al., 1978; Sussman et al., 1978). At high temperature, where the molecule is essentially in the random-coil form, the isolated resonances merge with the large central peak (Guéron & Shulman, 1975).

The relatively large ^{31}P shifts found for tRNAs are not observed for single- and double-stranded RNA and DNA oligonucleotides. For these model systems the ^{31}P resonance positions are located within the envelope of the main resonance found in tRNA spectra (line width at half-height is ~0.8 ppm). However, significantly downfield shifts were observed when actinomycin D was intercalated into double-stranded DNA, indicating that the position of resonances from the phosphate backbone depends on the conformation of the nucleic acid (Patel, 1976; Reinhardt & Krugh, 1977).

For assignment purposes we have studied the ^{31}P spectra of modified forms of yeast tRNA^{Phe}. The chemical or enzymatic modifications cause well-defined changes in the ^{31}P NMR spectra: individual, resolved resonances at high or low field from the main resonance either merge with the large resonance or shift to the terminal monoester or the 2′,3′-phosphate position. By comparing the spectra with the spectrum of the intact tRNA, we have been able to localize diester phosphates associated with resolved resonances within the tRNA structure. Conformational details of the tRNA backbone structure in solution are discussed in relation to the X-ray diffraction results. The degree of intactness of the modified tRNAs was estimated by using the corresponding low-field ^{1}H NMR spectra of the hydrogen-bonded protons.

Experimental Section

Materials. Yeast tRNA^{Phe} was purchased from Boehringer (batch no. 1236526) and used without further purification. The acceptance was 1.4 nmol of phenylalanine per A_{260} unit. No nicks were present in the sugar phosphate backbone as judged by ^{31}P NMR. Pancreas RNase A and RNase T_1 were purchased from Boehringer and Calbiochem, respectively. Sephadex G-100 was obtained from Pharmacia, and 99.75% pure D_2O was purchased from Merck. All other chemicals were analytical grade.

The chargeability of yeast tRNA^{Phe} and of its modification products was examined in a heterologous incorporation assay using partially purified *Escherichia coli* phenylalanyl-tRNA synthetase.

Instrumentation and Methods. The ^{31}P NMR spectra were recorded on a Varian XL-100 spectrometer, operating in the Fourier transform mode at 40.5 MHz. Heteronuclear proton noise decoupling was used to remove the J coupling induced by the ribose protons. A pulse width of 20 μs was employed, corresponding to a flip angle of 45°. Accumulation proceeded during 16–20 h with a spectral width of 2000 Hz and an acquisition time of 1 s; no pulse delay was used. Usually, a sensitivity enhancement was applied, yielding a line broadening of 0.6 Hz. The spectra were recorded at 33 or 35 °C. Re-

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ported chemical shifts are given relative to 20% H_3PO_4 as an external reference with downfield shifts defined as positive. tRNA samples were dissolved in 0.2 mL of D_2O buffer, containing 30 mM cacodylate, 80 mM NaCl, 1 mM EDTA, and 10 mM $MgCl_2$, at pH 7.0, unless specified otherwise. The D_2O solvent was used as an internal deuterium field frequency lock.

High-resolution ^1H NMR spectra were obtained on a Bruker 360-MHz spectrometer operating in the correlation spectroscopy mode (Dadok & Sprecher, 1974; Gupta et al., 1974). Scans of 2 or 3 s each were accumulated in a Nicolet BNC-12 computer. Unless specified otherwise, the buffers were the same as described for the ^{31}P experiments, except that H_2O solutions were used; spectra were recorded at 35 °C. Reported chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-5-sulfonate (DSS), with downfield shifts defined as positive.

Fourier transform infrared (FTIR) spectra were recorded at 20 °C on a Digilab FTS 15 BD spectrometer. Scans (200) of 1.5 s each were accumulated. The Michelson interferogram was Fourier transformed after phase correction in the time domain and application of a triangle apodization function.

Fluorescence spectra were recorded on a Perkin-Elmer MPF-4 fluorescence spectrophotometer.

Analysis of the enzymatically treated tRNAs was performed on a Sephadex G-100 column (1.7 × 150 cm) thermostated at 60 °C. Calibration of this column was carried out with five model systems that were used to construct an Andrews plot.

Modification Procedures. (*1*) *Cyanoethylation.* Cyanoethylation of yeast $tRNA^{Phe}$ was performed with acrylonitrile (Siddiqui et al., 1970). It has been shown that cyanoethylation occurs almost exclusively at the N_1 atoms of pseudouridines (Rake & Tener, 1966; Siddiqui et al., 1970). For yeast $tRNA^{Phe}$ these are Ψ_{39} and Ψ_{55} (see cloverleaf model, Figure 4). The reaction results in the formation of chemically stable N_1-(cyanoethyl)pseudouridine (Ofengand, 1967). The degree of cyanoethylation was measured by determining the loss of acceptor activity (Siddiqui et al., 1970). After termination of the modification reaction, the remaining chargeability amounted to about 8% with respect to intact tRNA, indicating that in 92% of the material pseudouridine had been converted. Incorporation of cyanoethyl groups could be confirmed from a comparison of FTIR spectra of the normal and the modified tRNA. Analysis of the modified tRNA by UV spectroscopy at 292 nm indicates that the m^7G_{46} base has been partially converted to an open-ring derivative, due to the alkaline conditions used for the cyanoethylation process.

(*2*) *Pancreas RNase Modification.* Endonuclease modification of yeast $tRNA^{Phe}$ with pancreas RNase A was performed using the following procedure. Yeast $tRNA^{Phe}$ (6.7 mg) was dissolved in 2 mL of buffer, containing 50 mM Tris-HCl and 20 mM $MgCl_2$ with pH 7.5, after which 41 μL of a pancreas RNase A stock solution in H_2O (0.4 mg/mL) was added. Subsequently, the tRNA solution was incubated at 17 °C for 100 min. Afterwards, the solution was quenched in ice, followed by two phenol extractions, using buffer-saturated phenol (Reid et al., 1972). Then, the tRNA was dialyzed against distilled water and subsequently lyophilized. Upon modification the chargeabiity of the tRNA decreased to 26%. The resulting material was analyzed with the calibrated Sephadex G-100 column at 60 °C. After modification, ~75% of $tRNA^{Phe}$ was converted to half molecules, i.e., nicked in the anticodon loop in accordance with expectation as will be discussed below; 10% of the tRNA has not reacted, while 15% has been digested further to one-quarter molecules.

(*3*) *RNase T_1 Modification.* Endonuclease modification of yeast $tRNA^{Phe}$ with RNase T_1 was carried out according to the following procedure. Yeast $tRNA^{Phe}$ (6.7 mg) was dissolved in 2 mL of buffer, containing 50 mM Tris-HCl and 20 mM $MgCl_2$ with pH 7.5, after which 37 μL of a RNase T_1 stock solution in H_2O (1.2 mg/mL) was added. Subsequently, the tRNA solution was incubated at 17 °C for 100 min. After this, the sample was treated in the same way as in the case of the pancreas RNase modification described above (Reid et al., 1972). After RNase T_1 modification the chargeability decreased to 33%. The resulting material was analyzed with the calibrated Sephadex G-100 column at 60 °C. After modification, a mixture of three-quarter and one-quarter tRNA molecules, produced by side-loop cleavage, was obtained and, in addition, small oligonucleotides with an average length of ~11 residues were obtained.

(*4*) *Excision of the Y Base.* Excision of the Y base was performed according to the procedure of Thiebe & Zachau (1968b); aniline hydrochloride was omitted from the reaction mixture in order to prevent scission of the polynucleotide chain. From fluorescence spectra it could be deduced that at least 87% of the Y base had been removed. Polyacrylamide gel electrophoresis showed that less than 16% of the tRNA was cleaved in the anticodon loop after Y-base removal. After Y-base removal the chargeability of the tRNA was reduced to 14%.

Results

The ^{31}P spectra of native tRNAs studied so far are characterized by one main large structured resonance and a number of well resolved resonances at high and low field with respect to the main resonance. This is true for yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{Glu}$ studied by Guêron & Shulman (1975) as well as for a number of other *E. coli* tRNAs (Salemink and Hilbers, unpublished experiments). The positions of the resolved resonances are sensitive to solution conditions but are very well reproducible when recorded under the same conditions. Below, ^{31}P spectra of intact yeast $tRNA^{Phe}$ under two solution conditions are studied, followed by a discussion of the effect of cyanoethylation, the effect of modification with pancreas RNase and RNase T_1, and the effect of removal of the Y base. Moreover, a correlation with ^1H NMR spectra of the hydrogen-bonded protons of these modified tRNA species will be made.

^{31}P Spectra of Intact $tRNA^{Phe}$. In Figure 1 a 40.5-MHz ^{31}P spectrum of intact yeast $tRNA^{Phe}$ is given. For convenience, the resolved resonances at high and low field have been lettered a–j. The relative intensity of these resonances was determined by integration, taking resonance b equal to unity, and by comparison of the experimental spectrum with a simulated spectrum. The simulated spectrum was obtained by convoluting 76 Lorentzian lines (based on the 76 phosphate groups present in the tRNA) by using line widths at a half-height of 3 Hz. The experimental and simulated spectra are compared in Figure 1. The resonances at high field tend to be somewhat broader than 3 Hz, giving a less perfect fit than the resonances at low field. The line width of the resonance from the terminal phosphate at ~3.5 ppm is always found to be somewhat smaller than 3 Hz, most probably due to its increased motional freedom in comparison with the diester phosphates. To give an impression of the distribution of the resonances, a stick spectrum is added to the simulated spectrum (Figure 1).

The intensities of the individual resonances obtained by these procedures are summarized in Table I. The resonances b, c, and e represent single diester phosphate groups and the

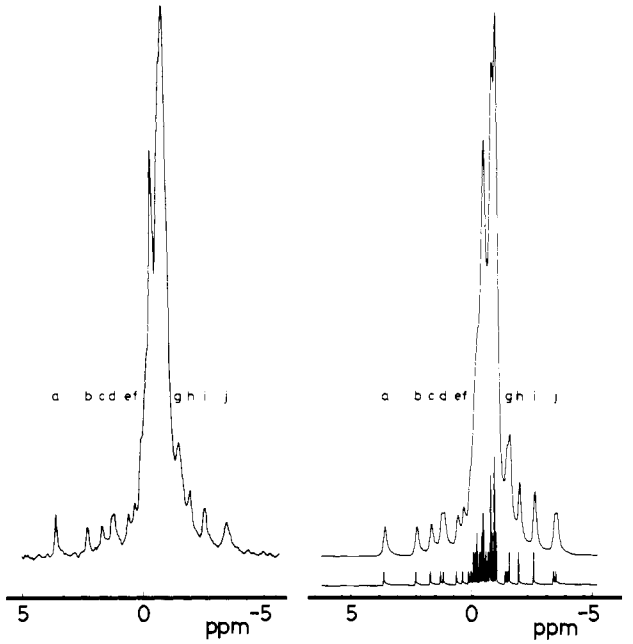

FIGURE 1: (Left) 40.5-MHz ^{31}P NMR spectrum of intact yeast tRNA^{Phe} in 30 mM cacodylate, 80 mM NaCl, 1 mM EDTA, and 10 mM $MgCl_2$ at pH 7 in D_2O, recorded at 33 °C. (Right) spectrum simulated with 76 Lorentzian lines, by using a line width at half-height of 3 Hz (see text).

Table I: Intensities of Resolved ^{31}P Resonances

modifcn of yeast tRNA^{Phe}	b	c	d_1	d	d_2	e	g + h	i	j_1	j	j_2
intact tRNA	1	1		2		1	7	2		2	
phenol extraction	1	1	1		1	1	7	2	1		1
cyano-ethylation	–	–	–		–	<1	–	–	–		–
pancreas RNase	1	–	1		1	–	9	2	1		–
RNase T_1	1	1	1		1	–	3	–	–		1
Y base	1	1		2		–	8[a]			1	

[a] Estimation for resonances g + h + i.

resonances d, i, and j correspond to two diester phosphates each. Resonances g + h together represent seven phosphate groups. Finally, the most low field resonance, a, is from a single phosphate which, as has been indicated above, can be assigned to the 5′-terminal phosphate. The estimated error in the intensity of the resolved resonances is ±20%, while that of the strongly overlapping resonances g and h corresponds to about one phosphate group. In the discussion below resonance f will not be taken into consideration because of its strong overlap with the main resonance. Differences in signal intensities due to nuclear Overhauser effects (NOE) have been neglected. It can be shown that for correlation times of $\tau_c \approx 10^{-8}$ s (estimated correlation time for tRNA) NOE hardly contributes to the signal intensity. The distance between the radio frequency pulses (1 s) is close to the longitudinal relaxation time T_1, and consequently, differences in intensities may arise if T_1 values for different diester phosphates are not the same. We expect these differences to be small except for the terminal phosphate.

After intact tRNA^{Phe} has been subjected to phenol extraction, as is necessary after enzymatic cleavage of the tRNA to stop the endonuclease reaction, some spectral changes are induced in the ^{31}P spectrum. This is shown in Figure 2, where the spectrum of the phenol-extracted tRNA is given together

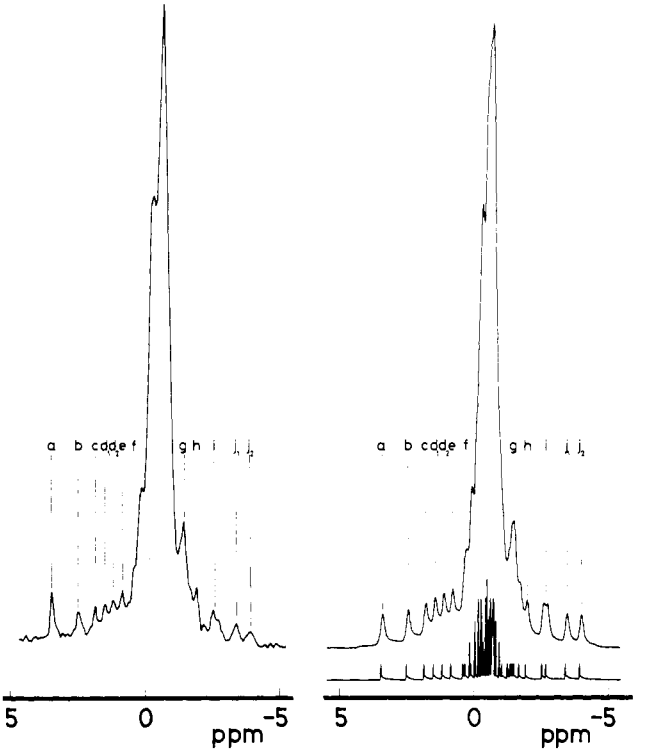

FIGURE 2: (Left) 40.5-MHz ^{31}P NMR spectrum of intact yeast tRNA^{Phe}, subjected to phenol extraction. Solution conditions were the same as those described in Figure 1. (Right) spectrum simulated with 76 Lorentzian lines, by using a line width at half-height of 3 Hz (see text).

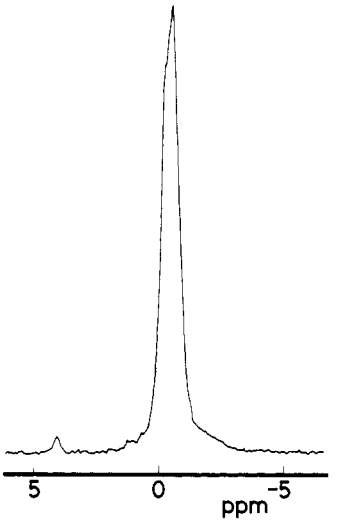

FIGURE 3: ^{31}P NMR spectrum (40.5 MHz) of cyanoethyl tRNA^{Phe}, dissolved in a solution containing 10 mM cacodylate, 100 mM NaCl, 10 mM $Na_2S_2O_3$, 10 mM $MgCl_2$, and 1 mM EDTA at pH 7 in D_2O. The spectrum was recorded at 35 °C.

with its simulated spectrum. The lettering of the resonances is an extension of that in Figure 1. Resonances d and j and to a smaller extent resonance i (Figure 1) are further resolved into single diester phosphate resonances (Figure 2). We do not know the reason for these spectral changes, but they nicely confirm the integration discussed above (see Table I). In conclusion, about 17 phosphate resonances are resolved from the main resonance in intact yeast tRNA^{Phe}.

Cyanoethylation. For the mild conditions under which the cyanoethylation reaction of Siddiqui et al. (1970) is performed, no cleavage of the phosphate backbone is expected to occur. Indeed, no resonances from cyclic ribose phosphate could be

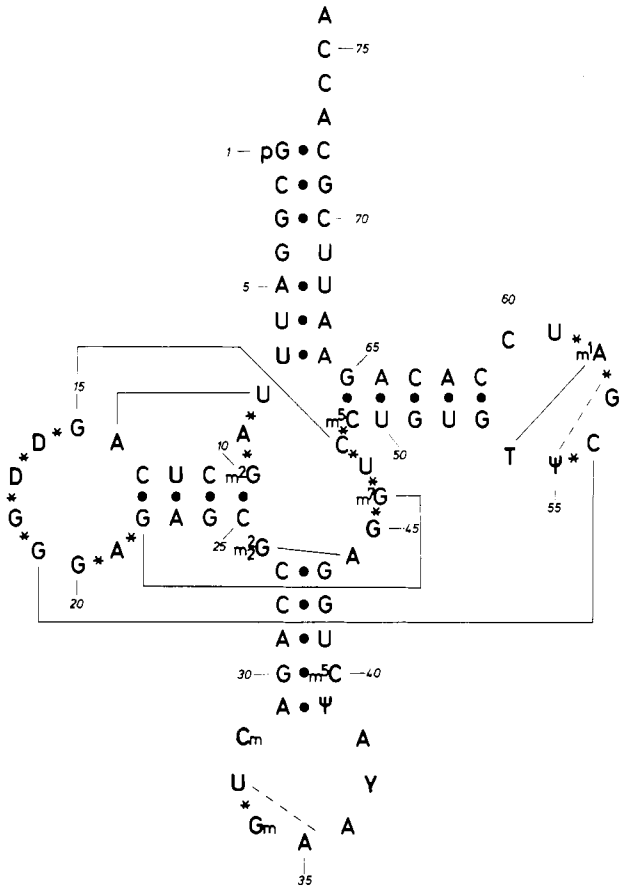

FIGURE 4: Cloverleaf structure of yeast $tRNA^{Phe}$ as determined by RajBhandary & Chang (1968). Asterisks indicate diesters with a deviating geometry (Jack et al., 1976). The dashed lines represent two base–phosphate interactions; the solid lines represent tertiary hydrogen-bond interactions expected to be visible in the low-field ^1H NMR spectrum.

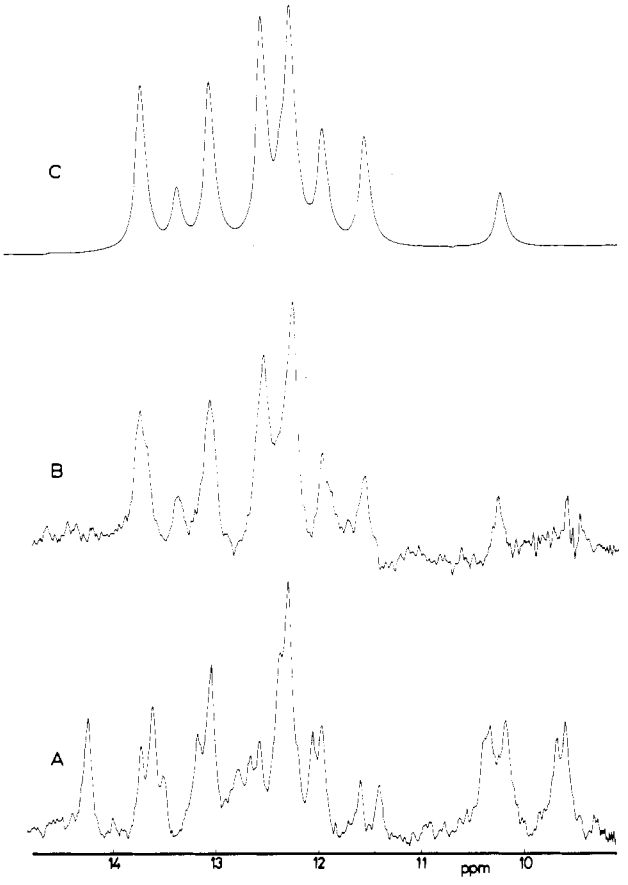

FIGURE 5: ^1H NMR spectra (360 MHz) of intact $tRNA^{Phe}$, 2000 scans (A), cyanoethyl-$tRNA^{Phe}$, 1200 scans (B), and a simulated spectrum (C). The tRNA was dissolved in a solution containing 10 mM cacodylate and 10 mM EDTA at pH 7 in H_2O. The spectra were recorded at 35 °C. The buffer for the intact tRNA also contained 10 mM $Na_2S_2O_3$.

observed in the ^{31}P spectrum of the cyanoethylated tRNA. Also, the relative intensity of the terminal-phosphate resonance at 4 ppm remained equal to that in intact tRNA within experimental accuracy (Figure 3).

As a result of modification of Ψ_{55} we expect the interaction between the DHU and the TΨC loop to be significantly weakened (see Figure 4). The interaction between G_{18} and Ψ_{55} found in the X-ray structure model will most likely be disrupted (Kim, 1976; Rich & RajBhandary, 1976; Ladner et al., 1975; Stout et al., 1976). This and the partial disruption of the m^7G_{46} base may affect the other tertiary interactions as well. This is demonstrated by the hydrogen bonded proton resonance spectrum given in Figure 5B. Comparison with the spectrum of the intact tRNA (Figure 5A) shows that it is quite different. The resonance at 14.3 ppm, equivalent with two hydrogen-bonded protons, has disappeared in the spectrum of the cyanoethylated material. One of the protons contributing to this resonance comes from the U_8-A_{14} tertiary base pair (Wong & Kearns, 1974; Reid et al., 1975; Daniel & Cohn, 1975), while there are some indications that the other proton contributing to this resonance comes from the T_{54}-m^1A_{58} tertiary base pair (Robillard et al., 1976b; Geerdes & Hilbers, 1977). At high field between 11 and 9 ppm most of the resonance intensity has disappeared in Figure 5B. Again this indicates that after modification tertiary structure has been disrupted. Most of the resonances in this spectral region are thought to come from exocyclic amino protons involved in hydrogen bonds (Reid et al., 1975; Steinmetz-Kayne et al., 1977).

If for the moment we assume that after modification we are left with $tRNA^{Phe}$ without tertiary structure, we expect to see a resonance of the GU pair in the acceptor stem around 10.5 ppm (Robillard et al., 1976a; Baan et al., 1977). Indeed, we find a resonance at 10.2 ppm, which by double-resonance techniques has been shown to come from the G_4-U_{69} pair in the acceptor stem (Johnston & Redfield, 1978). To estimate the relative intensity distribution of the resonances in Figure 5B, we simulated a spectrum by convoluting 21 Lorentzian lines expected from a $tRNA^{Phe}$ cloverleaf molecule (Figure 5C). The resonance at 10.2 ppm was taken to be equal to unity. In Table II the simulated positions are compared with resonance positions determined experimentally for yeast $tRNA^{Phe}$ on the basis of melting studies (Robillard et al., 1977), studies on hairpin fragments (Lightfoot et al., 1973; Rordorf, 1975), and double-resonance experiments (Johnston & Redfield, 1978). The base pair A_{31}-Ψ_{39} has been omitted, because it is assumed that this base pair is disturbed by cyanoethylation. The agreement of the resonance positions obtained in the present study with those from the literature studies is as good as can be expected for such a comparison. This and the proper intensity distribution in Figure 5B form strong evidence that the secondary structure has remained intact in the cyanoethylated tRNA.

The effect of cyanoethylation on the ^{31}P spectrum is shown in Figure 3. It is evident that all resonances b–j visible in Figure 1 have merged with the main resonance with the exception of some diffuse intensity left at ~1 ppm. Figure 3 very much resembles the ^{31}P spectrum of completely melted yeast $tRNA^{Phe}$, obtained by Guéron & Shulman (1975). Yet,

Table II

exptl position in cyano-ethylated tRNA (ppm)	intensity	exptl position based on lit. data[a]	assignment
13.6	3	14.0	AU_{12}
		13.7	AU_5
		13.7	AU_6
13.3	1	13.3	AU_{29}
		13.3	AU_{50}
13.0	3	13.3	GC_{11}
		13.0	AU_7
		12.7	GC_{10}
12.5	4	12.6	GC_{53}
		12.6	GC_{51}
		12.5	GC_{30}
		12.5	GC_{27}
12.3	1	12.4	GC_2
		12.3	GC_1
12.2	4	12.2	GC_{49}
		12.1	GC_3
11.9	2	12.0	GC_{28}
		11.8	GC_{13}
11.5	2	11.8	GU_4
10.2	1	10.4	GU_4

[a] The data were taken from melting studies (Robillard et al., 1977), fragment studies (Lightfoot et al., 1973; Rordorf, 1975), and double-resonance experiments (Johnston & Redfield, 1978). The base pair $A\Psi_{31}$, disturbed by cyanoethylation, has been omitted. A position for AU_{52} is not given because of discrepancies in the literature concerning this base pair. In regard to the base pair AU_{12}, it is noted that its resonance position in intact tRNA is predicted at ~13.8 ppm, i.e., 0.2 ppm closer to our observed position of 13.6 ppm.

as indicated above, the secondary structure of the tRNA has been retained after the modification procedure. Apparently, the loss of tertiary structure is associated with the disappearance of the resolved phosphate resonances.

Endonuclease Modification by Pancreas RNase A. Yeast tRNA^{Phe} was cleaved with pancreas RNase A, which is specific for pyrimidines in single-stranded regions. It has been shown that the ACCA terminus and the anticodon loop at the 3′-phosphate of U_{33} (i.e., phosphate 34) are most susceptible toward RNase A attack (Chang & RajBhandary, 1968; Reid et al., 1972), in agreement with the compact structure derived from X-ray diffraction studies (Kim, 1976; Rich & RajBhandary, 1976; Ladner et al., 1975; Stout et al., 1976). In the present experiments we have obtained similar results. About 75% of the tRNA has been converted into half molecules, i.e., nicked in the anticodon loop (see Experimental).

The ^{31}P NMR spectrum of this modified tRNA^{Phe} is presented in Figure 6A, where it is compared with a spectrum recorded for intact yeast tRNA^{Phe} (Figure 6C). The latter tRNA had been phenol-extracted analogously to the enzymatically treated tRNA^{Phe} sample (see also Figure 2). Comparison of the ^{31}P spectra of parts A and C of Figure 6 shows that the relative intensity of resonance a has increased. Its intensity corresponds to 6 ± 1 phosphates (calibrated with respect to resonances b, d_1, and d_2, whose combined areas have been taken equal to three phosphates). By cleavage of the ACCA terminus and by cleavage at U_{33}, four extra 3′-terminal phosphates are generated, superimposed on the resonance from the 5′-terminal phosphate at 3.5 ppm. Near 20 ppm (not shown in Figure 6) a resonance with an intensity of about unity is observed, arising from an intermediate hydrolysis product, i.e., ribose 2′,3′-phosphate. The intensity equivalent to approximately seven terminal-phosphate resonances is in

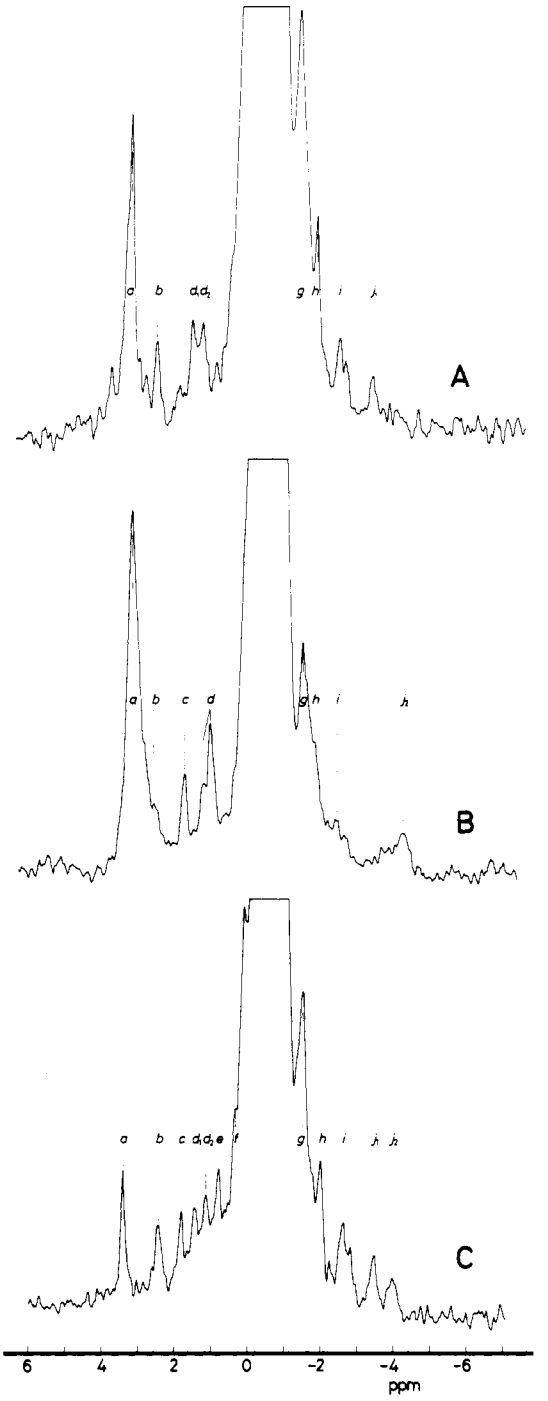

FIGURE 6: ^{31}P NMR spectra (40.5 MHz) of tRNA^{Phe} modified with pancreas RNase (A), tRNA^{Phe} modified with RNase T_1 (B), and phenol-extracted intact tRNA^{Phe} (C). The main resonance in each of the spectra has been cut off. The spectra were recorded at 33 °C; solution conditions were the same as those described in Figure 1.

quantitative agreement with the number of five terminal phosphates expected after modification, since it should be realized that resonances from the mononucleotides will exhibit an enhanced intensity due to the nuclear Overhauser effect. In addition to these changes, resonances c, e, and j_2 have disappeared. On the other hand, the positions of resonances b, d_1, d_2, g, h, i, and j_1 are virtually unaffected by the modification. The intensities of the resonances taking the intensity of resonance b as unity are presented in Table I.

It is interesting to see the effect of the modification reaction upon the hydrogen bonds in the tRNA. In Figure 7 the low-field ^{1}H spectrum of intact tRNA^{Phe} (Figure 7A) is

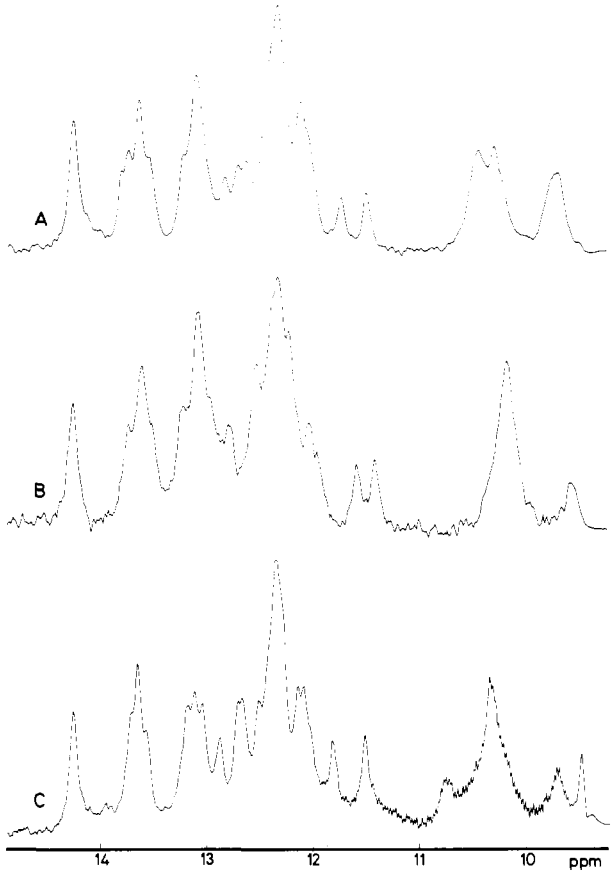

FIGURE 7: ^{1}H NMR spectra (360 MHz) of intact tRNA^{Phe}, 500 scans (A), tRNA^{Phe} modified with pancreas RNase, 800 scans (B), and tRNA^{Phe}–Y, 1900 scans (C). The spectra were recorded in H_2O with solution conditions the same as those described in Figure 1, except for the [$MgCl_2$] in Figure 7A, which was 17 mM. The spectra were recorded at 35 °C.

compared with that of the RNase A treated tRNA^{Phe} (Figure 7B). Between 11 and 13 ppm some changes in resonance positions are seen to occur, and at higher field, the resonance at 10.5 ppm is shifted to 10.3 ppm. However, we do not find a decrease in the number of ring N hydrogen bonds of the secondary and tertiary tRNA structure (11–15 ppm) after removal of the ACCA end and scission of the anticodon loop. On the basis of these results we conclude that the disappearance of ^{31}P resonances c, e, and j_2 has to be attributed to the disappearance of a special backbone folding in the anticodon loop.

Endonuclease Modification by RNase T_1. Ribonuclease T_1 specifically nicks after guanosines in single-stranded RNA. The anticodon loop of yeast tRNA^{Phe} is not susceptible to RNase T_1 digestion, since the only G residue present, G_{34}, is methylated on the 2′-OH group of its ribose. Hence, formation of ribose 2′,3′-phosphate, which is an intermediate in endonuclease T_1 fragmentation, is impaired. As a result, the fragments found after Sephadex G-100 chromatography arise from side-loop cleavage, which is followed by further digestion. Since the time constants for cleavage reactions of particular sites are not too different (Schmidt & Reid, 1971; Schmidt et al., 1970), we cannot expect such well-defined results as those obtained for the pancreas RNase modification.

In Figure 6B the ^{31}P spectrum of tRNA^{Phe} resulting from RNase T_1 treatment is presented. Comparison with the spectrum of intact tRNA^{Phe} (Figure 6C) shows that the relative intensity of resonance a has considerably increased as after treatment with pancreas RNase. Guanine 3′-phosphates are generated by side-loop cleavage and by production

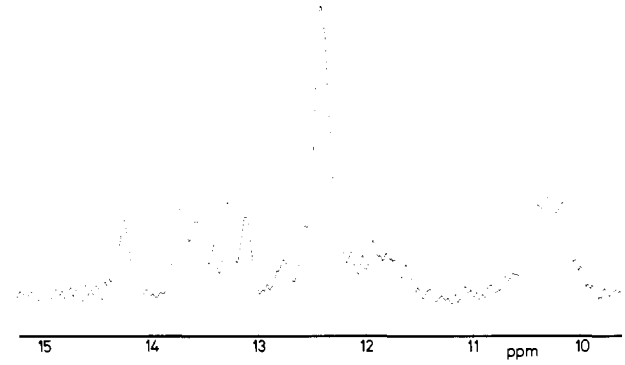

FIGURE 8: ^{1}H NMR spectrum (360 MHz) of RNase T_1 modified tRNA^{Phe} (2200 scans). The spectrum was recorded at 35 °C in H_2O with solution conditions the same as those described in Figure 1.

of smaller fragments. Also, resonances from ribose 2′,3′-phosphate are observed near 20 ppm (not shown). After the modification, the resonances d_1, d_2, and probably b remain although their positions are slightly affected. We can be less sure of resonance b because of overlap with the much larger resonance a. The resonances e, i, and j_1 have decreased to such an extent as to be hardly visible in the spectrum. The integration of the resolved resonances is given in Table I, taking resonance c equal to unity. Although the sample consists of a mixture of molecules, it is well-defined in the sense that the structured molecules, from which we are seeing the NMR spectrum, contain the intact anticodon branch. Indeed, the resonances c and j_2, lost after modification with pancreas RNase, remain after the RNase T_1 treatment, confirming their assignment to diester phosphates in the anticodon loop. On the other hand, the resonances i and j_1, lost after RNase T_1 treatment, persist in the spectrum of the pancreas RNase treated tRNA^{Phe}.

The 360-MHz hydrogen bonded proton NMR spectrum of the modified material is given in Figure 8. Comparison with the spectrum obtained after cyanoethylation (Figure 5B) suggests that more tertiary structure is retained after RNase T_1 treatment. In the latter case, a resonance at 14.3 ppm remains and also more resonance intensity is retained in the 11–9-ppm region compared to Figure 5B. On the basis of the chromatographic analysis of the RNase T_1 digest, we have to conclude that this ^{1}H spectrum is not merely a superposition of a cloverleaf structure plus residual tertiary structure. Extensive loop cleavage, e.g., in the DHU loop (four guanosines) or the TΨC loop, may have resulted in destabilization of secondary structure hydrogen bonds nearby. This and the fact that we are looking at a mixture of species cause the spectrum to be rather different from a normal yeast tRNA^{Phe} spectrum.

In conclusion, the ^{31}P resonances i and j_1, lost after RNase T_1 modification, most likely come from diester phosphates in the side loops.

Excision of the Y Base. The Y base was excised from the anticodon loop of yeast tRNA^{Phe} by chemical modification according to Thiebe & Zachau (1968b). From the fluorescence experiments it follows that at least 87% of the Y base is removed, while on the basis of electrophoresis measurements less than 16% of the tRNA is cleaved at the position of the Y-base (see Experimental).

In Figure 9 the ^{31}P NMR spectra of Y base deficient tRNA^{Phe} (Figure 9A) and intact tRNA^{Phe} not treated with phenol (Figure 9B) are compared. ^{31}P resonances b–d shift ~0.3 ppm upfield after Y-base removal. Resonance j decreases to single phosphate intensity, while resonance e shifts into the main resonance. A redistribution of resonances g–i

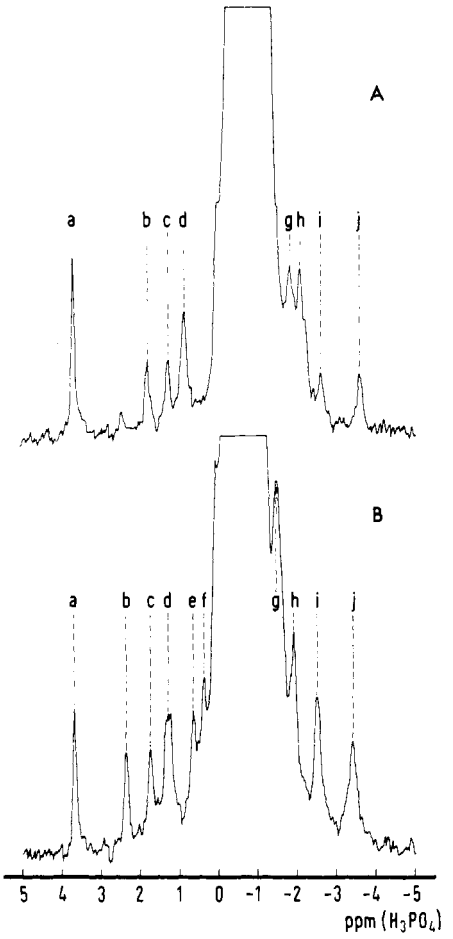

FIGURE 9: ^{31}P NMR spectra (40.5 MHz) of tRNA^{Phe}–Y (A) and intact tRNA^{Phe} (B). The spectra were recorded at 33 °C; solution conditions were the same as those described in Figure 1.

has taken place, but within experimental accuracy their total intensity has not changed. (For integration data see Table I.) The disappearance of resonance e and one resonance in j is consistent with the conclusion that these two resonances originate from the anticodon loop (see Endonuclease Modification by Pancreas RNase A). On the other hand, the presence of resonance c after Y-base removal would mean that some folding in the anticodon loop is preserved. This is reasonable, because the anticodon loop may still experience restrictions in its motion after Y-base removal, since the sugar phosphate backbone remains intact.

Figure 7C presents the low-field ^{1}H NMR spectrum of Y base deficient tRNA^{Phe}. Upon comparison with the ^{1}H spectrum of intact tRNA^{Phe} (Figure 7A), it is evident that one hydrogen bond is lost at 13.3 ppm, while some minor changes in position are present between 11 and 15 ppm. A probable assignment of the resonance lost at 13.3 ppm is the terminal base pair A_{31}-Ψ_{39} of the anticodon stem, which may be destabilized by the absence of the native structure of the anticodon loop. The spectral changes between 11 and 9.5 ppm are very similar to those observed for the pancreas RNase modification.

Discussion

^{31}P Spectra. Significant deviations from the normal diester phosphate geometry are found in a number of internucleotide regions in the crystal structure of yeast tRNA^{Phe}. Moreover, several diester phosphates are involved in hydrogen bonding (Jack et al., 1976; Sundaralingam et al., 1976; Kim & Sussman, 1976; Quigley & Rich, 1976; Stout et al., 1978; Hingerty et al., 1978; Holbrook et al., 1978; Sussman et al., 1978). These particular diester phosphates, located near invariant or semiinvariant residues involved in tertiary structure, are the most likely candidates to produce ^{31}P resonances, discernible from the regular diester position around 0 ppm (see Figure 4). Support for this comes from a number of observations.

In ^{31}P NMR melting experiments of tRNA, the well resolved resonances merge with the main resonance while proceeding from the native structure to the random-coil form (Guêron & Shulman, 1975). Intercalation of actinomycin D into double-stranded DNA results in downfield shifts of 1.5–2.5 ppm for DNA ^{31}P resonances, indicating a relationship between the ^{31}P chemical shift and the diester conformation (Patel, 1976). Similar observations were made by Reinhardt & Krugh (1977). Even more drastic effects are observed for the strained ribose 2′,3′-phosphate, produced, for instance, by alkali hydrolysis or endonuclease digestion, which resonates near 20 ppm downfield from H_3PO_4 (Weiner et al., 1974). Also, theoretical calculations give some indication of a correlation between the ^{31}P chemical shift of a phosphate ester and its torsional angles ω (P–$O_{5'}$) and ω' (P–$O_{3'}$) and its O–P–O bond angle θ (Gorenstein, 1975; Gorenstein & Kar, 1975; Gorenstein et al., 1976).

The present report confirms and extends the presumed relationship between the special diesters in yeast tRNA^{Phe} and the well resolved resonances in its ^{31}P spectrum. Ring-current calculations (Geerdes, unpublished calculations) show that the positions of these resonances cannot be accounted for by the ring currents from neighboring bases. The ^{1}H spectrum resulting after cyanoethylation can be accounted for by assuming that only the secondary structure is left after modification (Figure 5B). ^{31}P resonances b–j are shifted into the main resonance except for some residual intensity left at ~1 ppm (Figure 3). Therefore, we conclude that these resolved resonances are generated at their positions by the specific folding and/or hydrogen bonding of the phosphate backbone, imposed by the tertiary structure and the interactions within the loops. In this respect it is interesting to note that ^{31}P spectra of unfractionated yeast tRNA contain resolved resonances at positions close to those found in yeast tRNA^{Phe}, i.e., positions of resonances b, d, and j. This suggests a similar backbone conformation for the tRNAs in the mixture.

The other modification procedures, i.e., pancreas RNase and RNase T_1 modification and Y-base removal, lead to selective disappearance of resolved ^{31}P resonances. With some exceptions the different modification reactions perturb different ^{31}P resonances. RNase A leads to scission of the anticodon loop and removal of the ACCA terminus. For intact tRNA we expect this latter part of the molecule to be reasonably flexible in solution so that its phosphate groups will not contribute to the resolved ^{31}P resonances. By scission at U_{33} the anticodon loop is transferred into two more or less floppy ends, and thus one expects resolved ^{31}P resonances arising from special foldings in the anticodon loop to disappear. Indeed, the three resonances c, e, and j_2, present in intact tRNA^{Phe} (Figure 6C), are absent in tRNA^{Phe} treated with pancreas RNase (Figure 6A). Yet, after scission of the anticodon loop, the hydrogen-bonded framework of tRNA^{Phe} remains virtually intact as demonstrated by the hydrogen bonded proton spectrum (Figure 7B).

Resonances c and j_2 are retained in the ^{31}P spectrum after RNase T_1 treatment, which leads to side-loop cleavage but leaves the anticodon loop intact. Therefore, we conclude that these two resonances arise from two diester phosphates located

in the anticodon loop. We are less sure about resonance e, which disappears in both enzymatic modification procedures as well as upon removal of the Y base. The X-ray data of $tRNA^{Phe}$ show that the anticodon loop contains several special diester phosphates, i.e., phosphates with torsional angles deviating from the normal g^-,g^- conformation and one hydrogen-bonded phosphate (Stout et al., 1978; Hingerty et al., 1978; Holbrook et al., 1978).

The ^{31}P and ^1H spectra, obtained after RNase T_1 treatment (Figures 6B and 8), indicate that more of the tertiary structure has remained intact than is seen after the cyanoethylation reaction (Figures 3 and 5B). The ^{31}P resonances d_1 and d_2 as well as three out of a total of seven resonances from resonances g + h remain in the ^{31}P spectrum in addition to the resonances c and j_2 (see Table I). The residual tertiary structure after RNase T_1 treatment is also reflected by the ^1H resonance at 14.3 ppm. These observations point to the preservation of specially folded nucleotide regions inaccessible under our incubation conditions. We expect the nucleotide regions P_8–P_{10} and P_{44}–P_{49} to contribute to the ^{31}P resonances d_1, d_2, and g + h, since these parts of the molecule are relatively less susceptible to RNase T_1 than are the side loops (Schmidt et al., 1970; Reid et al., 1972). These results are consistent with recently published ω',ω plots (Jack et al., 1976; Hingerty et al., 1978) which contain six diesters with torsional angles deviating from the g^-,g^- conformation arising from these two segments (Figure 4).

The modification experiments in conjunction with the X-ray structure data in principle permit the assignment of some of the resolved ^{31}P resonances. However, this should be done with some caution, because different X-ray models disagree to some extent (Hingerty et al., 1978; Holbrook et al., 1978).

If the X-ray structure is preserved in solution, the special diester conformations present in the anticodon loop, i.e., P_{34}, P_{37}, and the hydrogen-bonded P_{36}, are expected to disappear upon scission at U_{33} (Hingerty et al., 1978). The ^{31}P resonances lost after this modification are located respectively at low and high field with respect to the main resonance. We expect resonance j_2 to come from the hydrogen-bonded P_{36}. The reason to expect an upfield shift for the hydrogen-bonded phosphate comes from NMR studies of the double-helical acid form of oligo(A), which contains diester phosphates in a g^-,g^- conformation hydrogen-bonded to adenine NH_2 groups (Rich et al., 1961); these studies reveal an upfield shift of 1.2 ppm with respect to phosphate resonances of single-stranded oligo(A) or 1.8 ppm upfield from external H_3PO_4 (Geerdes, 1979). In this context it should also be mentioned that protonation of diester phosphates results in upfield shifts (Cozzone & Jardetzky, 1976). The phosphate groups in the anticodon loop with a g^-,t geometry, i.e., P_{34} and P_{37}, are expected to resonate at low field, as exemplified by the disappearance of resonances c and e. The g^-,t geometry for P_{34} appears to be a common structural feature in the different X-ray structure models. Because a g^-,t geometry for diester phosphates P_{10} and P_{56} is also a common structural feature, we also expect P_{10} and P_{56} to resonate at low field [see also Patel (1976)].

From the present results in combination with literature data, we arrive at the conclusion that deviations from the g^-,g^- conformation as a result of bending or stretching and hydrogen bonding cause shifting of the diester phosphate resonances. It has been pointed out by Jack et al. (1976) that bending mainly occurs by rotating ω ($P–O_{5'}$) from g^- to t or by changing the sugar pucker on the 5′ side to $C_{2'}$ endo or by a combination of both. Stretches, however, are introduced by rotating ω' ($P–O_{3'}$) to t or by changing the sugar pucker on the 3′ side to $C_{2'}$ endo. It is to be expected that ^{31}P shifts are not sensitive to the sugar pucker (Cozzone & Jardetzky, 1976). Also, the resonance positions for the phosphate groups in double-helical DNA and double-helical RNA are close to 0 ppm, i.e., within the envelope of the main resonance in tRNA, while their sugar conformations are $C_{2'}$ endo and $C_{3'}$ endo, respectively. Consequently, changes in the torsional angles ω and ω' and the O–P–O angle θ will largely determine the shift. From the discussion above, we can then conclude that bending leads to downfield shifts. Torsional angles not incorporated in this category like g^-,g^+ are also found in tRNA crystals. The phosphate in 3′,5′-cUMP has a g^-,g^+ conformation, and its resonance is shifted 1.6 ppm upfield from H_3PO_4 (Cozzone & Jardetzky, 1976). This indicates that g^-,g^+ conformations in tRNA may resonate upfield from H_3PO_4. Therefore, proceeding from 3 to −4 ppm we expect ω',ω conformations of diester phosphates to resonate in the following order: g^-,t; g^-,g^-; g^-,g^+. Finally, upfield shifts are expected upon hydrogen bonding.

^1H Spectra. It is now well established that the resonances between 15 and 11 ppm obtained for yeast $tRNA^{Phe}$ under the salt conditions in Figure 7 comprise signals from the secondary as well as from the tertiary ring N hydrogen-bonded protons. The modifications in the anticodon loop, i.e., nicking after U_{33} and removal of the Y base, produce some minor changes in the hydrogen bonded proton spectra (see Figure 7). In addition, Y-base modification results in the loss of one resonance at 13.3 ppm most likely from the AΨ pair in the anticodon stem. The position is in good agreement with that found earlier in fragment studies and melting experiments (Shulman et al., 1973; Hilbers et al., 1973). Thus, apart from disrupture of and/or changes in the anticodon loop structure, these modifications leave the secondary and tertiary structure of the tRNA molecule virtually unchanged as far as the ring N hydrogen-bonded protons are concerned.

It is interesting to compare these results with some earlier studies of the structure of tRNA in solution. On the basis of the hydrogen bonded proton NMR studies of four different anticodon hairpins, Kearns (1977) suggested that a common ^1H resonance near 11.5 ppm arises from the $U_{33}N_3H$–P_{36} hydrogen bond present in the anticodon loop. We expect this interaction to disappear after scission at P_{34} or removal of the Y base. Yet, the resonance at ~11.5 ppm in the yeast $tRNA^{Phe}$ spectrum pertains in these two cases. Therefore, we think that at least for this tRNA the suggestion does not hold.

It is well established that after Y-base removal yeast $tRNA^{Phe}$ can no longer be charged with *E. coli* phenylalanyl-tRNA synthetase (Thiebe & Zachau, 1968a). Homologous charging can still be achieved after Y-base removal, although with a 1 order of magnitude lower affinity (Krauss et al., 1976). The low-field ^1H spectrum of $tRNA^{Phe}$–Y (Figure 7) strongly suggests that upon modification structural changes are mainly induced in the anticodon-loop region, possibly including the terminal AΨ base pair. Therefore, the conclusion seems to be justified that the native structure of the anticodon loop, maintained by the Y base, is essential for the reaction with *E. coli* phenylalanyl-tRNA synthetase. However, on the basis of the ^{31}P spectrum of $tRNA^{Phe}$–Y (Figure 9A), small changes in backbone conformation, which are not manifest in ^1H NMR, possibly occur in more distant parts of the molecule, e.g., as exemplified by the 0.3 ppm upfield shifts for resonances b and d. The latter observation is not inconsistent with a reported effect of Y-base excision upon the conformation of the entire anticodon stem (Kearns

et al., 1973), which was reversed after substitution with proflavine (Wong et al., 1975).

Finally, the foregoing discussion also demonstrates that ^{31}P NMR yields valuable and necessary information, supplementary to ^{1}H NMR data, to give a more complete description of structure not easily obtainable otherwise.

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