Unfolding of Yeast Transfer Ribonucleic Acid Species Caused by Addition of Organic Solvents and Studied by Circular Dichroism†

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ABSTRACT: Denaturation of five highly purified yeast tRNAs including $tRNA^{Phe}$ caused by addition of organic solvents has been studied by means of circular dichroism and ultraviolet absorption techniques. Even at low concentrations, organic solvents cause structural changes so that tRNA is partially denatured under most commonly used crystallization conditions. Those organic solvents can, under certain conditions, stabilize a highly structured tRNA conformation as indicated by the anisotropic factor.

There are many elaborate ways of crystallizing tRNA (Ladner *et al.*, 1972; Cramer *et al.*, 1970, 1974; Kim *et al.*, 1971). These methods usually have in common that the tRNA is dissolved in a buffer and then an organic solvent is added as precipitant.

In this paper we report ultraviolet (uv) and circular dichroic (CD) spectroscopic investigations of the denaturation of five highly purified yeast tRNAs caused by addition of organic solvents. The fact that tRNA is partially unfolded under commonly used crystallization conditions is of particular interest with respect to the recent X-ray crystallographic analysis of $tRNA^{Phe}$ (Kim *et al.*, 1973a) and is pertinent to the question of whether the tertiary structure of native tRNA can be deduced from crystals grown under these conditions. Naturally, some previous investigations showed the way in which to proceed. Circular dichroism has been used as an indicator for configurational changes in tRNA (Willick *et al.*, 1973). Unfolding of tRNA caused by organic solvents has been observed previously by nuclear magnetic resonance (nmr) (Schweizer, 1969). There also have been CD and optical rotatory dispersion (ORD) investigations on conformational changes of DNA and polydeoxynucleotides in water and organic solvents (Green and Mahler, 1971; Ivanov *et al.* 1973).

Materials and Methods

Purified phenylalanine, tyrosine, serine, valine, and isoleucine tRNAs from yeast were prepared according to Schneider *et al.* (1972). They had a specific amino acid acceptance per A_{260} unit of tRNA of 1.55, 1.46, 1.43, 1.2, and 1.15 nmol of amino acid, respectively. One A_{260} unit of tRNA corresponds to 1.67, 1.65, 1.53, 1.64, and 1.65 nmol of the particular tRNA as determined by phosphate analysis. $tRNA^{Phe}$ obtained from Boehringer, Mannheim, Germany, had a specific amino acid acceptance of 1.03 nmol/A_{260} unit.

Dioxane and 2-methyl-2,4-pentanediol were obtained from Fluka, Buchs, Switzerland. Spermine and spermidine were from Serva, Heidelberg, West Germany. All other reagents including 2-butanol, ethanol, and 2-propanol were purchased from Merck, Darmstadt, West Germany. Pyridinium-*N*-phenolbetaine was a gift from C. Reichardt, Marburg, Germany.

In order to reduce the cation contamination to below 10^{-4} M, dialysis was performed in a microdialysis chamber (Neuhoff and Kiehl, 1963). $tRNA^{Phe}$ was dialyzed against EDTA, the concentration of which was reduced in ten steps from 10^{-1} to 10^{-4} M over a period of 8 hr. The final step was dialysis against water (6 hr). This denaturation was reversible.

Routine absorbance measurements were performed with a Zeiss PMQ II and a Unicam SP 1800 spectrophotometer. Uv melting curves were measured as described previously (Maelicke *et al.*, 1973).

CD measurements were performed with a Cary 61 spectropolarimeter equipped with a thermostatically controlled cuvet holder. The temperature was set to 22° unless specifically stated. Selected Hellma fluorescence cuvets with 1-cm path length were used. Temperature was measured in the stoppered sample cells by means of a Pt-100 resistor lead thermometer, suspended in the solution just above the light path. A Haacke temperature programmer was used to establish a linear temperature increase of 18°/hr.

Results

Conformational Changes of tRNA Caused by Increasing Temperature. The temperature melting of $tRNA^{Phe}$ from yeast can be followed by its CD spectrum (Figure 1). Two stages of melting can be distinguished in the presence of Mg^{2+}: firstly, up to temperatures of about 70°, there is a slight decrease in the overall CD signal. Secondly, there is a sharp decrease in the CD signal combined with a pronounced red shift of the 263-nm maximum and a characteristic change of the whole spectrum. The first stages of melting may be detected more readily by the CD melting curve of 260 nm than by the uv melting curve at this wavelength even though the relative changes are very similar (Figure 2).

Anisotropic Factor g. To study denaturation of tRNA by organic solvents a tRNA sample was placed in a cuvet which contained the desired mixture of organic solvents and buffer. Each cuvet could be used for measuring the ellipticity θ as well as the uv absorption A (see Materials and Methods). The anisotropic factor g ($g = \theta/A$) of ellipticity $\theta = [\theta]cd$ and uv absorption $A = \epsilon cd$, both measured at the same wavelength

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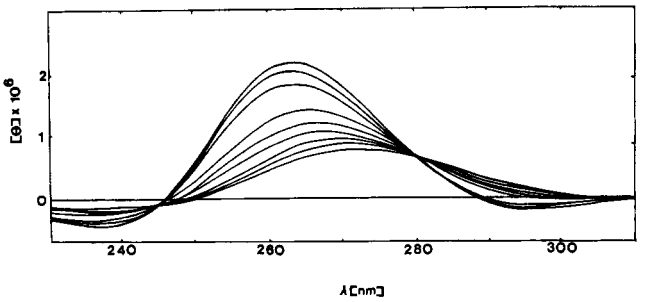

FIGURE 1: CD spectra of $tRNA^{Phe}$ at 28, 45, 65, 70.1, 70.7, 73.2, 75.5, and 78.5°. Buffer conditions: 0.2 M NaCl, 0.01 M $MgSO_4$, and 0.01 M cacodylate (pH 7.0).

was used as a parameter to detect denaturation of tRNA by organic solvents. Since θ/A of the same sample and in the same cuvet is independent of concentration, the choice of g as a measure of denaturation avoids concentration errors. The errors are then due to spectroscopic techniques alone. They are approximately 3%.

A melting curve for g (Figure 3) exhibits the same characteristics as the melting curve for $[\theta]$.

Spectral Changes under Crystallization Conditions. Under all of the investigated crystallization conditions (Table I) at least a partial melting of tRNA was observed. For each g value of $tRNA^{Phe}$ a temperature can be deduced from Figure 3 which under physiological conditions (conditions 1 in Table I) would correspond to analog distributions of native and unfolded states. For example, a $tRNA^{Phe}$ solution containing 0.01 M $MgCl_2$, 0.001 M spermine hydrochloride, and 0.01 M cacodylate (pH 6.0) in the aqueous phase (95%) and 5% 2-methyl-2,4-pentanediol (conditions 9 in Table I) at 4° gave $g = 0.0308$. This corresponds to 92.1% of native g (conditions 1 in Table I) and a temperature of 51° in the melting curve of native $tRNA^{Phe}$ (conditions 1 in Table I). The percentage of g (in relation to conditions 1 in Table I) cannot be correlated quantitatively to a change in structure. In particular a reduction by, say, 10% does not mean that 10% of the bases are unstacked. The corresponding temperature also has no meaning in itself, but it gives a rough estimate as to how much $tRNA^{Phe}$ is affected by the crystallization conditions. The other tRNA samples investigated were affected more. Serine specific tRNA, for example, showed the CD spectrum of its unfolded state under most of the crystallization conditions. This agrees well with the fact that we have not been able to crystallize this tRNA in the presence of organic solvents.

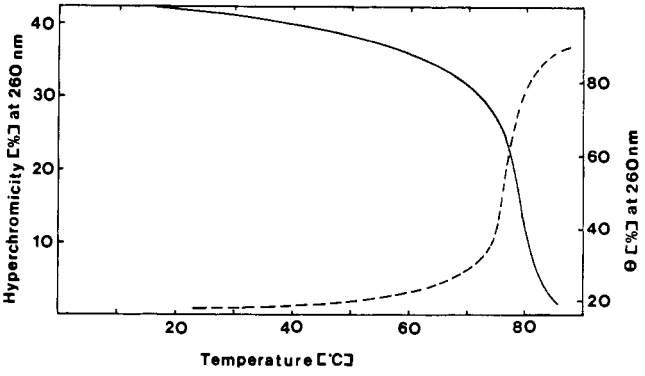

FIGURE 2: CD (—) and uv (- - -) temperature melting curves of $tRNA^{Phe}$ at 260 nm. Uv temperature melting curve in per cent hyperchromicity *vs.* temperature, CD melting curve in per cent hypoellipticity *vs.* temperature. Buffer conditions see Figure 1.

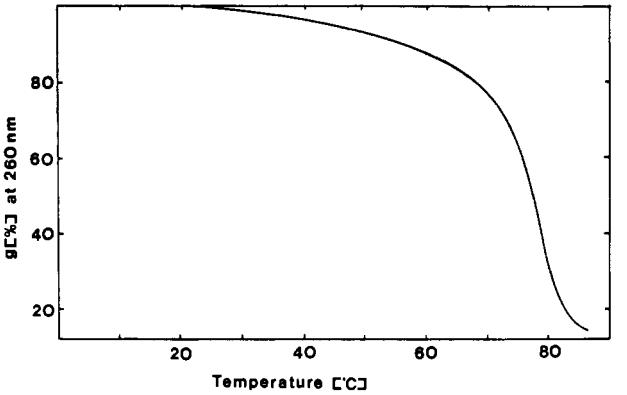

FIGURE 3: g (%) at 260 nm *vs.* temperature of $tRNA^{Phe}$. The g value at 20° is taken as 100%. Buffer conditions see Figure 1.

That tRNA can be crystallized in the absence of organic solvents under conditions which exhibit an identical g value as the native conformation has been shown by Cramer *et al.* (1974), who crystallized $tRNA^{Tyr}$ in an aqueous solution which was 50% saturated with ammonium sulfate and contained 0.01 M $MgSO_4$. $tRNA^{Phe}$ also crystallizes in a solution containing 1.5×10^{-2} M $MgCl_2$, 1.25×10^{-2} M spermine hydrochloride, and 10^{-3} M sodium cacodylate (pH 7.0) (Cramer *et al.*, 1974).

Changes of tRNA Conformations by Organic Solvent–Buffer Mixtures. In order to gain more insight into the conformational changes caused by addition of organic solvents, CD spectra were studied over a wide concentration range of organic solvents and ions. The tRNA was introduced into a series of cuvets containing a varying ratio of organic solvent and buffer and the denaturation of tRNA could be monitored by changes in the CD spectra (Figure 4a). These changes in the CD spectra were very similar to those spectral changes observed upon thermal denaturation (Figure 1). The unfolding took place in a narrow concentration range of the organic solvent. With an aqueous phase containing 0.2 M NaCl, 10^{-4} M Mg^{2+}, and 0.01 M cacodylate (pH 7.0), the midpoint of this

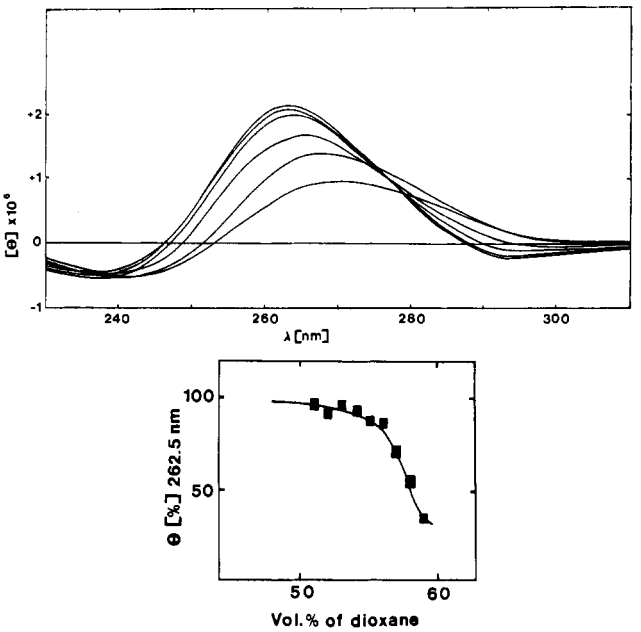

FIGURE 4: (a, top) CD spectra of $tRNA^{Phe}$ at 51, 52, 55, 57, 58, and 59% of dioxane added to an aqueous buffer of 0.2 M NaCl, 10^{-4} M Mg^{2+}, and 0.01 M cacodylate (pH 7.0). (b, bottom) θ (%) at 262.5 nm *vs.* increasing ratio of dioxane. Same experiment as part a.

TABLE I: g Values at 260 nm for Purified Yeast tRNAs under Various Crystallization Conditions.

Solvent Conditions[a]	$tRNA^{Phe}$			$tRNA^{Ser}$		$tRNA^{Val}$		$tRNA^{Ile}$	
	g	%[b]	Temp (°C)[c]	g	%	g	%	g	%
1	0.0334	100	22	0.0363	100	0.0283	100	0.0242	100
2	0.0222	66.5	74	0.0107	29.5	0.0117	41.2		
3	0.0281	84.0	64.5	0.0223	61.5	0.0228	71.5	0.0166	68.6
4	0.0292	87.5	59.5	0.0284	78.0	0.0250	88.1	0.0201	83
5	0.0306	91.6	54	0.0180	49.5	0.0248	87.5	0.0214	88.5
6	0.0304	91.0	55.2						
7	0.0294	88.0	59						
8	0.0306	91.6	52.5						
9	0.0308	92.1	51.0						
10	0.0317	95.0	45						

[a] Solvent conditions: (1) 0.2 M NaCl, 0.01 M $MgSO_4$, and 0.01 M cacodylate, pH 7.0, 22°; (2) 0.01 M $MgCl_2$, 1.5 mM spermidine hydrochloride, 1 mM cacodylate, pH 7.0 (80%), and dioxane (20%), 22° (Ladner *et al.*, 1972); (3) 0.01 M $MgCl_2$, 0.01 M spermidine hydrochloride, 0.01 M $SrCl_2$ (80%), and 2-methyl-2,4-pentanediol (20%), 22° (Cramer *et al.*, 1974); (4) aqueous phase as in 3 (90%), 2-butanol (10%), 22° (Cramer *et al.*, 1974); (5) 0.01 M $MgCl_2$, 1 mM spermine hydrochloride, 0.01 M cacodylate, pH 6.0 (90%), 2-methyl-2,4-pentanediol (10%), 22°; (6) aqueous phase as in 5 (95%), 2-methyl-2,4-pentanediol (5%), 22°; (7) aqueous phase as in 5 (90%), 2-propanol (10%), 22°; (8) aqueous phase as in 5 (90%), 2-methyl-2,4-pentanediol (10%), 4° (Kim *et al.*, 1971); (9) aqueous phase as in 5 (95%), 2-methyl-2,4-pentanediol (5%), 4° (Kim *et al.*, 1971); (10) $tRNA^{Phe}$ without Mg^{2+}, 0.2 M NaCl and 0.1 M cacodylate, pH 7.0, 22° (volumes in volume per cent). [b] The g value under solvent conditions 1 is taken as 100%, all other g values of the same tRNA sample were related to this value. [c] Temperatures corresponding to each g value of $tRNA^{Phe}$ deduced from the temperature melting curve in Figure 3.

transition, P_m (in analogy to the melting point T_m), was at approximately 57% of dioxane for $tRNA^{Phe}$ (Figure 4b). Other highly purified tRNAs behaved in a similar way (Table II). The midpoints P_m of all of these transitions depended on the ion content of the aqueous phase.

Denaturation was also observed with methanol, ethanol, 2-propanol, 2-butanol, 2-methyl-2,4-pentanediol, and *N*-methylformamide. As a rule, the P_m value did not depend so much on the particular organic solvent than on the ion content of the aqueous phase (expt 1–6 of Table III). A particular aqueous buffer (expt 7 of Table III) with 0.2 M NaCl required 54% of ethanol to unfold the $tRNA^{Phe}$ structure. Replacing NaCl by 0.67 M $MgCl_2$ (expt 8 of Table III) destabilized the tRNA even though the ionic strength was kept constant. In this case only 42% of ethanol was required to unfold the structure. Doubling the NaCl content had almost no effect. Spermine and spermidine did behave similarly as Mg^{2+}.

The question arises which of the physical parameters of the solvent are crucial for the denaturation. The dielectric constant, ϵ_{25}, cannot be responsible for the denaturation because *N*-methylformamide (ϵ = 182.4) as well as dioxane (ϵ = 2.21) cause unfolding. Water (ϵ = 78.5) leaves it intact when there are small amounts of salt present.

TABLE II: Midpoint P_m (%, v/v, Dioxane in Solvent) of Purified tRNA Unfolding Caused by Dioxane at 22°.

Solvent Conditions[a]	$tRNA^{Phe}$	$tRNA^{Ile}$	$tRNA^{Ser}$
1	57	57	54
2	37	32	28

[a] Solvent conditions: (1) 0.2 M NaCl, 10^{-4} M $MgSO_4$, and 0.01 M cacodylate, pH 7.0; (2) 0.2 M NaCl, 10^{-4} M $MgSO_4$, 0.01 M spermidine hydrochloride, and 0.01 M cacodylate, pH 7.0.

The pH value of the buffer increases only moderately with addition of organic solvent so that the denaturation is not related to a sudden increase in pH.

The transition energy E_T of the uv absorption of pyridinium-*N*-phenolbetaine is an empirical parameter related to the polarity of solvents (Dimroth *et al.*, 1963; Reichardt, 1965). This parameter was determined for the solvent conditions P_m

TABLE III: Midpoint P_m (%, v/v, Organic Solvent of Total Solvent) of Unfolding $tRNA^{Phe}$ from Boehringer Caused by Organic Solvents and Transition Energies E_T of Pyridinium-*N*-phenolbetaine at the Solvent Conditions of P_m.

	Solvent	P_m (%)	E_T (kcal/mol)
1	Dioxane–H_2O	95	40
2	2-Propanol–H_2O	90	50.4
3	Ethanol–H_2O	90	52.7
4	Dioxane–0.01 M cacodylate, 0.2 M NaCl, 0.001 M $MgCl_2$, pH 7	59	52.7
5	2-Propanol–same buffer as in 4	53	53.4
6	Ethanol–same buffer as in 4	59	54.8
7	Ethanol–0.01 M cacodylate, 0.2 M NaCl, 0.01 M $MgCl_2$, pH 7.5	54	55.4
8	Ethanol–0.01 M cacodylate, 0.4 M NaCl, 0.01 M $MgCl_2$, pH 7.5	53	55.4
9	Ethanol–0.01 M cacodylate, 0.077 M $MgCl_2$, pH 7.5	42	56.3
10	2-Propanol–0.01 M cacodylate, 0.2 M NaCl, 0.01 M $MgCl_2$, 0.01 M spermidine·HCl	40	55

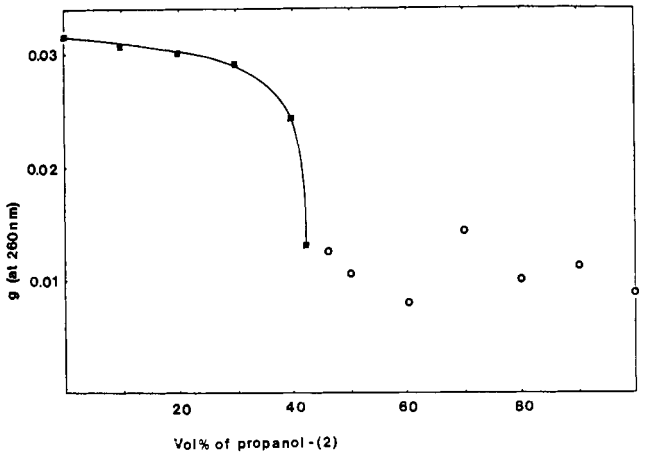

FIGURE 5: g of $tRNA^{Phe}$ from Boehringer at 260 nm *vs.* increasing concentration of 2-propanol. Aqueous buffer contained 10^{-2} M cacodylate, 0.2 M NaCl, 10^{-2} M $MgCl_2$, and 10^{-2} M spermidine hydrochloride (pH 7.0). (O) Turbidity in the cuvets.

of Table III. If polarity of solvent would be the decisive parameter for denaturation, one would expect an equal E_T for all P_m independent of the chemical nature of the solvent. This is not the case (Table III).

Plotting the anisotropic factor g *vs.* an increasing ratio of organic solvent gave in the presence of a certain buffer a continuous curve up to concentrations of organic solvents that were so high that the tRNA was unfolded (Figures 5 and 6). At higher concentrations of organic solvents there were discontinuities which were hardly reproducible. They may have resulted from precipitation of tRNA as there usually was some turbidity in the cuvets. However, replacing the buffer by water gives rise to quite a different configurational change of tRNA (Figure 7). $tRNA^{Phe}$ which had been dialyzed against water showed the CD spectrum of an unfolded conformation (configuration 2 of Figure 8). Adding 2-propanol had the effect of increasing the anisotropic factor θ/A to a value which would correspond to native tRNA (Figure 7). However, the full CD spectrum was different from the native one (configuration 1 of Figure 8). The ratio $\theta_{min\ 237}/\theta_{max\ 263}$ of the minimum $\theta_{min\ 237}$ at 237 nm and the maximum $\theta_{max\ 263}$ at 263 nm was reduced by about a factor 2 compared to the CD spectrum of native tRNA. The same change in the CD spectrum was observed using 80% dioxane or ethanol instead

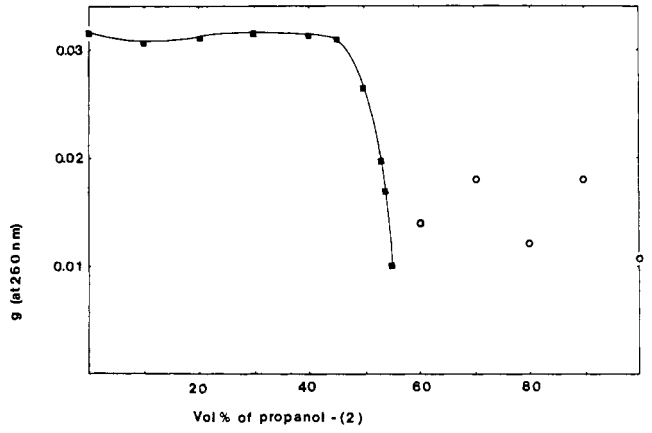

FIGURE 6: g of $tRNA^{Phe}$ from Boehringer at 260 nm *vs.* increasing concentration of 2-propanol. Aqueous buffer contained 10^{-2} M cacodylate, 0.2 M NaCl, and 10^{-3} M $MgCl_2$ (pH 7.0). (O) Turbidity in the cuvets.

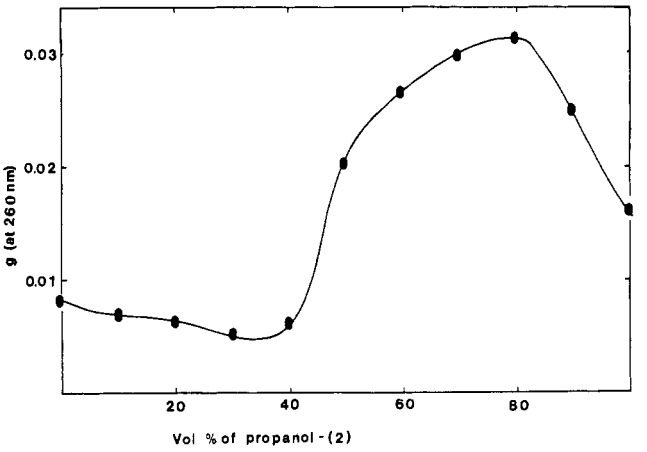

FIGURE 7: g of $tRNA^{Phe}$ at 260 nm *vs.* increasing concentration of 2-propanol. The tRNA had been dialyzed extensively against water. Aqueous phase: H_2O.

of 2-propanol. $tRNA^{Phe}$ from Boehringer gave similar results as dialyzed highly purified $tRNA^{Phe}$.

With nondialyzed $tRNA^{Phe}$ a change from configuration 3 of Figure 8 to configuration 1 of Figure 8 was observed at 70% 2-propanol and 30% H_2O. Varying the 2-propanol content did not show much variation of the anisotropic factor g, but g had a minimum at 40% 2-propanol and 60% H_2O.

Denaturation of tRNA by Urea. Contrary to its response to organic solvents, no initial stages of melting were detected by adding small amounts of urea to $tRNA^{Phe}$. Even at concentrations of 1 M urea in standard buffer (conditions 1 of Table I) $tRNA^{Phe}$ was in its native conformation judging from its CD spectra and g values. Denaturation began at about 5 M urea. At 9 M urea $tRNA^{Phe}$ was completely denatured.

Discussion

tRNA can be denatured by addition of organic solvents. We now find that the amount of organic solvent needed to unfold tRNA depends mainly on the salt content of the aqueous phase. The nature of the particular organic solvent is of minor importance. This can be explained in the following way. The three-dimensional structure of tRNA is, among other forces, stabilized by hydrophobic interactions. A change in the water

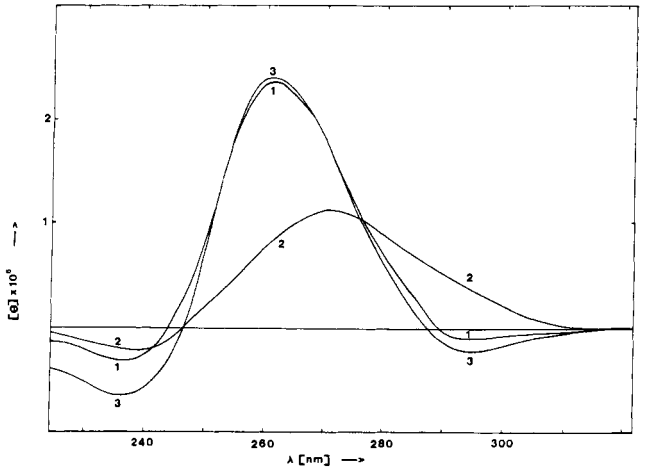

FIGURE 8: CD spectra of $tRNA^{Phe}$ that had been dialyzed against water: solvent 1, 80% 2-propanol and 20% H_2O; solvent 2, H_2O (100%); solvent 3, aqueous buffer containing 10^{-2} M cacodylate–0.2 M NaCl–10^{-2} M $MgSO_4$.

structure will affect these interactions. This change can be caused by addition of organic solvents. It will be enhanced by high ionic strengths, because ions will bind a considerable amount of water molecules in solution.

Unfolding by organic solvents is of particular interest with respect to the solvent conditions applied to crystallize tRNA. A certain degree of unfolding has been found under all of the conditions investigated (Table I). It was also found (Figures 7 and 8) that organic solvents can stabilize a nonnative conformation of $tRNA^{Phe}$ which has a highly organized structure according to its anisotropic factor. Thus one must look upon the prospect of crystallizing tRNA in its native conformation in the presence of organic solvent with some reservation. The observed exceptional degree of polymorphism of $tRNA^{Phe}$ crystals (Kim *et al.*, 1973b,c; Cramer *et al.*, 1974) might well result from different ways of refolding from partially unfolded tRNA. The tRNA conformation which forms the first seeds will obviously determine the conformation of all tRNA molecules in these crystals. Thus, tRNA crystals might grow in which from the basic cloverleaf structure different tertiary structures are formed.

Acknowledgments

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