General Procedure for Crystallization of Transfer Ribonucleic Acid*

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ABSTRACT: Simple reproducible conditions have been developed for crystallizing tRNAs from ethanol–water and aqueous ammonium sulfate solutions in both the presence and absence of various polyvalent cations. The nature of the polyvalent cation, its concentration, concentration of tRNA, and concentration of ethanol or ammonium sulfate are all important variables for obtaining large single crystals. The generality of these procedures has been shown by successful crystallization of both $tRNA^{Phe}$ from *Escherichia coli* and formylmethionine tRNA from yeast. Attempts to use these conditions for crystallization of seven other purified tRNAs were not successful, indicating the importance of slight variations in solution parameters as they affect successful tRNA crystallization. These crystals retained their biological activity when redissolved and assayed for the acceptance of amino acids. Although the crystals must be grown and maintained at 8–10°, conditions for handling and mounting them in capillaries to give X-ray diffraction data to 10-Å resolution have been determined. These results show the ease and reproducibility in obtaining large single crystals of tRNA for X-ray diffraction studies and the effects of solution parameters on their growth.

Transfer ribonucleic acid has been crystallized from both ethanol–water and aqueous ammonium sulfate systems. Two different tRNAs from two different species were crystallized, giving evidence of the generality of this method. These were $tRNA^{Phe}$ from *Escherichia coli* and $tRNA^{fMet}$ from yeast. In designing a procedure for crystallization of tRNA, the probability of success is greatly increased by a systematic approach. In order for crystals to form, it is necessary to have solutions in which the tRNA is near saturation with respect to amorphous precipitation. If ordered interactions of the type which build crystal lattices occur, the crystal will be less soluble than the amorphous precipitate. Because building of the highly ordered matrix of a crystal can be aided by any strong but highly selective intramolecular interactions, it is essential to test as many varied conditions as the amount of sample available permits. The variables likely to promote strong intramolecular interactions in tRNA are: (i) *polyvalent cations:* variations in valence, coordination number, ionic radius, and concentration were explored; (ii) *pH:* bases with pK's near 7 and near 4 are of particular concern in these studies, and pH ranges which affect ionization of such bases but are compatible with RNA stability were explored; (iii) *precipitating solvent:* solvents tested varied in concentration, dielectric constant, and hydrogen-bonding tendencies; and (iv) *monovalent cation:* concentration, ionic radius, and hydrogen-bonding tendency are important parameters, and potassium, cesium, ammonium, and tetraalkylammonium cations are of particular interest in tRNA structure.

In this research, particular emphasis was placed on the polyvalent cation variable because of known data on the interaction of these ions with both the phosphate groups and the heterocyclic bases of the tRNA. Considerable work in studying the interaction of metal ions with nucleic acids has been done (Wacker and Vallee, 1959; Butzow and Eichorn, 1965; Eichorn, 1967). Studies of the effects of metal binding on T_m have shown that metals have diverse specificities for either phosphate groups or the bases. Copper(II) and mercury(II) bind to the heterocyclic rings (Yamane and Davidson, 1961; Eichorn and Tarien, 1967). Magnesium(II) and cobalt(II) have a high ratio of affinity for the phosphates relative to the bases, while manganese(II) has a lower ratio of affinity. Spermine and spermidine bind primarily to phosphate groups (Hirschman *et al.*, 1967) as do chromium(III) and lead(II).

Because of the differences in affinity of these heavy metals for nucleic acids, it is likely that tRNA would have specific sites available which preferentially bind these polyvalent cations (Lindahl *et al.*, 1966) and thus allow the cation to behave as a specific electrostatic cross-linker. For this reason, all of the aforementioned polyvalent cations were tested in the crystallization system with a metal-to-phosphate molar ratio in the range of one-sixth. This ratio was kept well below one in an attempt to make the interaction selective rather than general.

Materials and Methods

General Methods. In principle, the method used to crystallize tRNA was an exchange of vapor within a sealed container between a large solvent reservoir and very small samples containing the tRNA. In the case of crystallization with

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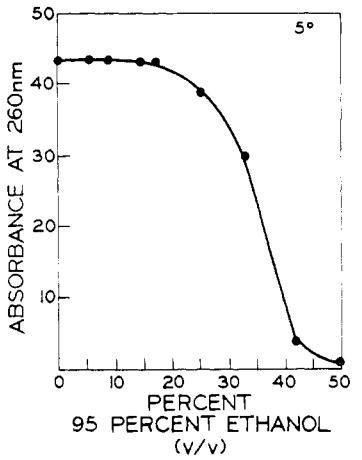

FIGURE 1: Solubility curve of partially purified tRNA. Partially purified $tRNA^{Gly}$ was dissolved in 0.005 M $MgCl_2$, 0.05 M KCl, and 0.005 M Tris·HCl at pH 7.4. After addition of 95% ethanol to the desired concentration, samples were left for 36 hr at 5°. The precipitate was removed, and the absorbancy of the supernatant at 260 nm was determined in a Cary 15 spectrophotometer.

ethanol as the precipitating agent, small increments of ethanol were added to a solvent reservoir and the vapor was allowed to diffuse into sample drops containing the tRNA, causing the ethanol concentration in these drops to increase very slowly. For ammonium sulfate, the tRNA samples and reservoir were prepared as 30% saturated ammonium sulfate. Increments of saturated ammonium sulfate were then added to the solvent reservoir, whereupon the concentration of ammonium sulfate in the drop increased very slowly as water was transported from the drops to the reservoir solution by vapor exchange. Three to five days was allowed for equilibration to take place, after which the samples were examined microscopically in the chamber with a dissecting microscope using 40× magnification. If no change had taken place in the samples, the concentration of precipitating agent was increased, and if precipitation had occurred, a decrease was made in the concentration of precipitating agent in the solvent reservoir.

The buffer used in the crystallization system contained 0.005 M Tris·HCl (pH 7.4) and 0.05 M KCl. Latt and Sober (1967) have shown that potassium and Tris cations have negligible specificity for poly (A + U) or poly (I + C), whereas sodium and magnesium cations display a greater binding preference for poly (A + U) than poly (I + C). Thus, to avoid preferential interactions with specific polynucleotide sequences, Tris was chosen as the buffer and potassium as the primary monovalent cation.

$tRNA^{Phe}$ from *Escherichia coli* B was supplied by the Oak Ridge National Laboratory and is described in publications by Kelmers (1966). The purity of this tRNA was approximately 60% as measured by moles of phenylalanine accepted per mole of terminal adenosine in the sample. Purified formylmethionine tRNA from yeast was provided by Raj-Bhandary (RajBhandary and Ghosh, 1969).

The tRNA was prepared for crystallization studies by precipitating with two volumes of 95% ethanol for 2 hr at −20°. The precipitate was redissolved in 0.1 M KCl and 0.01 M Tris·HCl at pH 7.4. The tRNA was precipitated and redissolved by this procedure two more times before either being dried with a dry stream of N_2 gas or lyophilized to dryness and then dissolved in the buffer to the desired concentration.

All salts were reagent grade except for spermidine and spermine, which were Aldrich Nos. 5382 and 5383, respectively. The 95% ethanol was obtained from Commercial Solvents Co., Chicago, Ill.

Construction of Chambers for Vapor Equilibration. The crystallization chambers were designed to allow storage of a large number of samples in a small area under controlled temperature. The chambers permitted easy addition of samples to depressions on microscope slides within the chamber and addition of large volumes of solvent to a reservoir whose vapors equilibrated with the droplets of sample. In addition, the dimensions of the chamber were such that low-power microscopic observations could be conducted without opening the chamber.

These chambers were constructed by grinding flat the upper lip of a Pyrex pie plate (9 in. diameter) and constructing a 0.25-in. thick Lucite sheet to cover the chamber. The Lucite sheet had several small stoppered ports for addition of sample and solvents. Within the pie plate was a 7-in. disk of Lucite supported on 1-cm high legs of Lucite, which allowed the samples to be placed above the large liquid reservoir. Samples were placed in the depression of single cavity slides (3 × 1 in.) with a cavity 1.75 mm in depth and 18 mm in diameter, obtained from Schaar Scientific Co., Chicago, Ill. These had been treated with dichlorodimethylsilane to render the depression in the slides nonwetting. The nonwetting surface permitted the droplets to be reduced in volume as solvent diffused from droplets to the equilibration reservoir and prevented formation of a crust or film of dried buffer at the edge of the droplet.

Procedures for Growing Single Crystals of tRNA. Before attempts were made to crystallize tRNA, data were obtained to determine its solubility at various concentrations of the precipitating agent. The solubility of partially purified $tRNA^{Gly}$ in various concentrations of 95% ethanol at 5° was determined. These data served as an initial guide for approaching the critical solubility range in the crystallization samples (Figure 1).

Data on ammonium sulfate solubility were taken directly from the published work of Lindahl and Fresco (1967) on mixed tRNA. All stock solutions of ammonium sulfate used in this study were saturated at 5°.

Initially, 22 different samples were prepared for crystallization studies: 11 samples for the ethanol–water system and the corresponding 11 samples for the aqueous ammonium sulfate system. The only difference between these two series was that the samples for the ammonium sulfate system were all made up to 30% saturation with ammonium sulfate, while none was added to the samples in the ethanol–water system. A summary of the common components in each sample tested is given in Table I.

Results

Solubility of tRNA. The solubility of a partially purified sample of $tRNA^{Gly}$ in aqueous ethanol is shown in Figure 1. It can be seen that the amount of tRNA remaining in solution as measured by its absorbance at 260 nm decreases very

TABLE I: Composition of Crystallization Samples.[a]

Sample Designation[b]	$MgCl_2$ Concn (M)	Additional Added Salt[c]
1	0.01	
2	0.005	Spermidine
3	0.005	Spermine
4	0.005	$HgCl_2$
5	0.005	$PbCl_2$
6	0.005	$CoCl_2$
7	0.005	$MnCl_2$
8	0.005	$CuCl_2$
9	0.005	$CrCl_3$
10	0.001	
11		

[a] See text for details. [b] All samples contained 0.05 M KCl–0.005 M Tris·HCl of pH 7.4 in addition to tRNA. [c] All are 0.001 M.

rapidly when the concentration of 95% ethanol is greater than about 20% v/v.

Crystal Growth of E. coli tRNA^{Phe} in Aqueous Ethanol Solutions. Into each cavity of 11-cavity slides/chamber was added 0.02 ml of the respective solution described in Table I containing tRNA^{Phe} with a final absorbancy of 92 at 260 nm. Into the solvent reservoir were added 42 ml of water with incremental additions of 95% ethanol such that the precipitation level was approached over a period of a few weeks. The chambers were stored in a cold room in an area which remained at 8–10°. After 10.5 ml of 95% ethanol had been added to the plate, five samples precipitated amorphously and one crystallized. Amorphous precipitates formed in samples 1, 2, 3, 5, and 8, while sample 6 containing cobalt(II) crystallized. The amorphous samples were placed in a plate with a lower ethanol concentration in the reservoir. Samples 1 and 2 crystallized when the ethanol was lowered to 7.4 ml of 95% ethanol while using 42 ml of water as before in the crystallization chamber. Sample 10 crystallized into a very blocky crystal when the 95% ethanol was raised to 11 ml in the solvent reservoir, and sample 11 crystallized into a diamond shaped crystal with 11.8 ml of 95% ethanol in the reservoir. In all of these cases, the water volume in the chamber was 42 ml. Attempts to crystallize samples 3, 4, 5, 7, 8, and 9 failed as the samples either remained in solution or formed an amorphous precipitate when changes in ethanol concentration were made. A few attempts to repeat crystal growth from conditions 10 and 11 have been unsuccessful. Conditions 1 and 2 have led to crystals several times; however, condition 1 has a nonuniform growth habit (Figure 2) which makes it difficult to use for X-ray diffraction studies. Consequently, no further work was done with this crystal.

Sample 6, crystallized from 0.001 M $CoCl_2$, 0.005 M $MgCl_2$, 0.05 M KCl, and 0.005 M Tris·HCl (pH 7.4) and tRNA with absorbancy 92 or 98 at 260 nm, has been found to crystallize very reproducibly from a chamber containing 42 ml of water and 11 ml of 95% ethanol. These samples grow very rapidly into large, well-formed, single crystals in 5 days (Figure 3A,B).

FIGURE 2: Photomicrograph of tRNA^{Phe} crystals grown in the presence of 0.01 M $MgCl_2$ and ethanol (procedure as described in the text). Note the extreme crystalline deformations and lack of discrete faces or edges on the crystals (ruled scale = 0.02 mm/division). All photomicrographs in this report were taken through a Unitron crystallographic microscope using a Zeiss photomicrograph attachment fitted with a Model 500 Polaroid Land film holder using Polaroid Black and White Type No. 57, 3000-speed, 4 × 5 in. film packets. In each case, the photograph of the crystal was taken while it remained surrounded by mother liquor.

The crystalline form is a hexagonal prism with pointed ends and usual dimensions of 0.2 mm in diameter and 0.8 mm in length. These crystals are strongly birefringent and extinguish sharply between crossed Polaroids. While in the mother liquor, they are also very thermolabile and sensitive to slight losses in ethanol. When crystals in the drop of mother liquor are placed at 25°, they disappear completely in approximately 15 min, or if they are left exposed to the atmosphere for periods of about 10 min at the same temperature at which they were grown, they also begin to disappear. Consequently, these crystals must be handled and manipulated at 8–10° in an atmosphere of controlled ethanol vapor.

Samples containing cobalt chloride with various initial concentrations of tRNA have been found to crystallize differently. When the tRNA^{Phe} was lowered to an absorbancy of 62 at 260 nm, crystals were not as well formed as before (Figure 3C). There was a greater tendency to form satellite crystals from the main crystal which was, in general, smaller. Initial tRNA^{Phe} concentrations of 135 A_{260} units/ml gave few usable single crystals. In general, the results were as shown in Figure 3D where many crystals are found connected together, making it difficult to work with one single crystal.

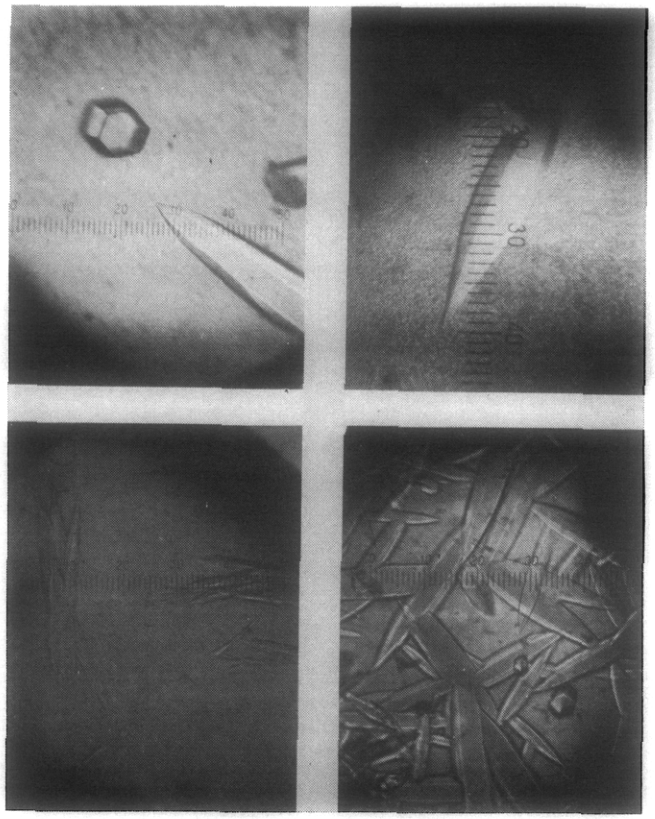

FIGURE 3: Crystals of *E. coli* $tRNA^{Phe}$ grown in the presence of cobalt chloride and ethanol (procedure as described in the text). (A) Upper left: a crystallization sample with a $tRNA^{Phe}$ concentration of 92–98 A_{260} units/ml shows one crystal growing perpendicularly to the bottom of the cavity slide and another crystal growing longitudinally, both showing the nature of the hexagonal prism (scale = 0.02 mm/division). (B) Upper right: a large single crystal grown from a sample containing 98 A_{260} units/ml of $tRNA^{Phe}$ (scale = 0.06 mm/division). (C) Lower left: a sample containing 62 A_{260} units/ml of $tRNA^{Phe}$ showing many de-formed crystals (scale = 0.02 mm/division). (D) Lower right: a sample containing 135 A_{260} units/ml of $tRNA^{Phe}$. The crystals are connected together, and very few single unattached crystals are present (scale = 0.02 mm/division).

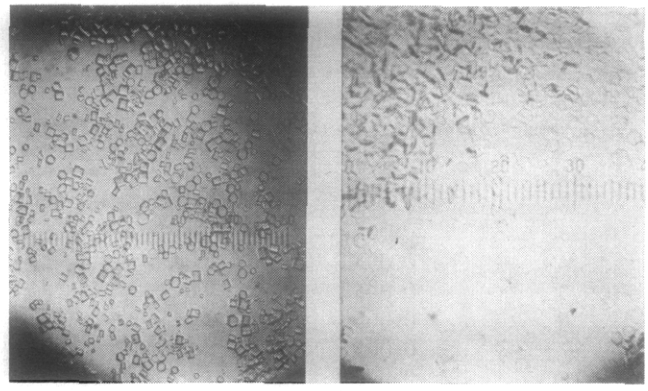

FIGURE 4: Crystals of yeast $tRNA^{fMet}$ grown in the presence of 0.001 M $CoCl_2$ and ethanol (procedure as described in the text). The crystals are in the form of hexagonal prisms. (A, left) Crystals with a diameter-to-height ratio of about 1:1, and (B, right) crystals of a much flatter nature with a diameter-to-height ratio of about 5:1 (scale = 0.02 mm/division).

At $tRNA^{Phe}$ concentrations higher than 135 A_{260} units/ml, this network became even more pronounced as more and more crystals overlapped.

Experiments wherein the concentration of magnesium chloride and cobalt chloride were raised 75% for the $tRNA^{Phe}$ sample having an absorbancy of 135 at 260 nm yielded crystals of the same shape and habit as before with X-ray diffraction patterns showing similar resolution.

Crystal Growth of Yeast $tRNA^{fMet}$. $tRNA^{fMet}$ was found to crystallize from a solution containing 0.001 M $CoCl_2$, 0.005 M $MgCl_2$, 0.05 M KCl, and 0.005 M Tris·HCl of pH 7.4. Initial samples crystallized from a $tRNA^{fMet}$ solution with an absorbancy of 59 at 260 nm with a solvent reservoir of 42 ml of H_2O and 11 ml of 95% ethanol. The crystals were hexagonal prisms (Figure 4A) with a diameter-to-height ratio of approximately 1:1 and without pointed ends. These crystals showed no defects of types often present in $tRNA^{Phe}$ crystals. After the tRNA crystals remained in the mother liquor for periods of 1 week or more after growth, the number of clustered or de-formed $tRNA^{Phe}$ crystals increased, whereas no change occurred in $tRNA^{fMet}$ crystals.

Reproducible growth of large, useful, single crystals of $tRNA^{fMet}$ was difficult to accomplish. After several successful initial crystallizations, later crystallizations formed flat crystals with a diameter-to-height ratio of about 5:1 (Figure 4B). These flat crystals were not useful for X-ray diffraction studies because of their extremely short prism length. Varying the RNA concentration, temperature of growth, and salt-to-RNA ratio has not improved the length-to-width ratio of $tRNA^{fMet}$.

Crystallization of tRNA from Ammonium Sulfate Solutions. The general procedures used to crystallize tRNA from aqueous ammonium sulfate solutions were basically the same as those used for the ethanol–water system. The same fundamental solutions listed in Table I were used. In addition, all samples initially contained 30% saturated ammonium sulfate as did the solvent reservoir. The only samples to crystallize were 6 and 8, which contained $CoCl_2$ and $CuCl_2$, respectively. These resulting crystals were not true crystals but spherulites instead (Oppenlander, 1968). The photographs in Figure 5A–C were all taken through crossed Polaroids. The differences in birefringence indicate differences in order within the crystals. Figure 5A shows true spherulites with the characteristic extinction cross (Keith, 1963). These were grown in 0.001 M $CoCl_2$, 0.005 M $MgCl_2$, 0.05 M KCl, and 0.005 M Tris·HCl buffer of pH 7.4 with an ammonium sulfate concentration increasing from 30 to 58% saturation. The ammonium sulfate solutions were saturated at 5° and growth was at 8.5°. The material shown in Figure 5B has regions of crystalline orientation, and Figure 5C shows material which is ordered internally to give a single angle of rotation for each crystal. These were grown in the same way as those in Figure 5A except that the $CoCl_2$ was replaced by $CuCl_2$ and the ammonium sulfate concentration was varied from 30 to 53%. Because of their spherical habit of growth, these crystals have not yet proven useful for single crystal X-ray diffraction studies (Clark *et al.*, 1968). Recent modifications of these conditions have produced large crystals for $tRNA^{fMet}$ from yeast and $tRNA^{Leu}$ from *E. coli* (Young, 1969).

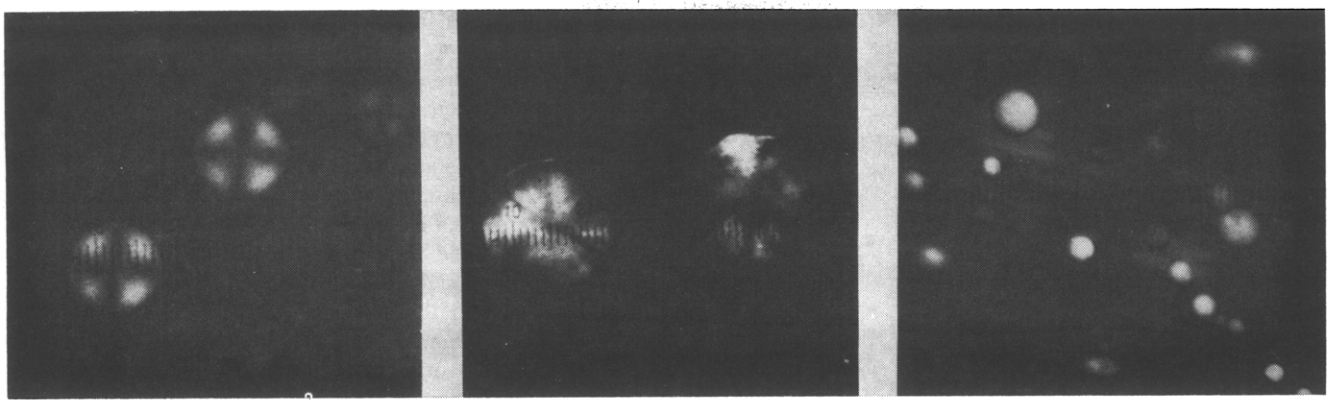

FIGURE 5: Spherulites of $tRNA^{Phe}$ grown from ammonium sulfate solutions. The photomicrographs were taken through crossed Polaroids (ruled scale = 0.02 mm/division). (A, left) True spherulites with the characteristic extinction cross, (B, middle) partially ordered structures, and (C, right) internally ordered spherulites, with some being extinguished and others showing birefringence.

Results with Other Purified and Partially Purified tRNAs. Several other purified and partially purified tRNAs have been tested in these crystallization systems. Attempts to crystallize them using methods previously discussed for $tRNA^{Phe}$ and $tRNA^{fMet}$ failed. Ten partially purified samples of tRNA from *Saccharomyces lactis* strain Y-14 ranging in purity from 2.5 to 44%, obtained from chromatographic columns run by Kirkegaard (1969), were set up in crystallization chambers exactly as before in a 0.02-ml drop containing 0.001 M $CoCl_2$, 0.005 M $MgCl_2$, 0.05 M KCl, and 0.005 M Tris·HCl (pH 7.4). They were carefully carried through the critical range of ethanol where precipitation of tRNA just begins to start. Five of the samples formed precipitates, and the other five formed an oily layer on the bottom of the drop. The samples and results are listed in Table II.

Several other purified samples of tRNA were tested similarly in the ethanol–water system in the presence of cobalt. $tRNA^{Tyr}$ and $tRNA^{Gly}$ both from *S. lactis* (Hampel, 1969; Kirkegaard, 1969) were both used in the crystallization system. The $tRNA^{Tyr}$ had a minimum purity of 85% as determined by amino acid acceptor activity, and $tRNA^{Gly}$ gave a single peak in the absorbancy profile on a high-resolution DEAE-Sephadex column and had a minimum purity of 50% as determined by amino acid acceptor activity (Hampel, 1969). Several attempts to crystallize these samples failed as $tRNA^{Tyr}$ precipitated each time and $tRNA^{Gly}$ formed an oily layer with occasional indication of structures in the oil.

Four purified samples of *E. coli* tRNA from Dr. S. Nishimura,[1] which were pure as judged by chromatographic patterns of a T-1 ribonuclease digest, also failed to give crystals in the presence of cobalt and ethanol. These were $tRNA^{Leu}$, $tRNA_I^{Val}$ (for GUG and GUA), $tRNA_{II}^{Tyr}$, and $tRNA^{fMet}$. The first three gave a precipitate, and $tRNA^{fMet}$ consistently formed an oily layer. Two other tRNAs from *E coli* were also tested. These were $tRNA^{Val}$ and $tRNA_1^{fMet}$ obtained from the Oak Ridge National Laboratory (Drury, 1968). $tRNA^{Val}$ accepted 1082 pmoles of amino acid/A_{260} unit; and $tRNA_1^{fMet}$ accepted 1328 pmoles of amino acid/A_{260} unit thus, the purities were approximately 65 and 80%, respectively. Under the crystallization conditions, $tRNA^{Val}$ precipitated and $tRNA_1^{fMet}$ formed an oil. These two samples were also tested in the ammonium sulfate system. The sample solution used was 0.001 M $CuCl_2$, 0.005 M $MgCl_2$, 0.05 M KCl, and 0.005 M Tris·HCl (pH 7.4), and $tRNA^{Val}$ of absorbancy 65 at 260 nm or $tRNA_1^{fMet}$ of absorbancy 82 at 260 nm. As the concentration of ammonium sulfate was brought to the critical level where crystallization or precipitation should occur, precipitation occurred in both cases.

A sample of $tRNA^{Cys}$ from Boehringer yeast was obtained from Dr. G. Tener.[2] This sample was pure as judged by amino acid acceptor assay and precipitated rather than crystallized in the ethanol system in the presence of cobalt.

TABLE II: Attempts to Crystallize Partially Pure tRNA in the Ethanol–Water System.[a]

Primary Amino Acid Accepted	Min Purity of Sample (%)	$A_{260\ nm}$ Used in Crystzn Samples	Result
Arginine	2.5	74	Oil
Lysine	5	78	Oil
Arginine	10	65	Oil
Methionine		67	Oil
Phenylalanine	12.2	67	Oil
Tyrosine	8	78	Oil
Glycine	9	78	Precipitate
Aspartic acid	4	55	Precipitate
Aspartic acid	3	58	Precipitate
Glycine, alanine		75	Precipitate
Isoleucine	44	77	Precipitate
Histidine	5.1	98	Precipitate

[a] The procedure is as described in the text.

[1] National Cancer Center Research Institute, Tokyo, Japan.

[2] The University of British Columbia, Vancouver, British Columbia, Can.

TABLE III: Attempts to Crystallize Purified tRNA in the Ethanol–Water System.

Amino Acid Accepted and tRNA Species	Sample Source	$A_{260\ nm}$ Used in Crystzn Samples	State of RNA Formed
Tyrosine (yeast)	Kirkegaard	80	Precipitate
Glycine (yeast)	Hampel	58	Oil
Leucine (*E. coli*)	Nishimura	70	Precipitate
Valine (*E. coli*)	Nishimura	74	Precipitate
Tyrosine (*E. coli*)	Nishimura	66	Precipitate
Formylmethionine (*E. coli*)	Nishimura	68	Oil
Valine (*E. coli*)	Oak Ridge	65	Precipitate
Formylmethionine (*E. coli*)	Oak Ridge	82	Oil
Cysteine (yeast)	Tener	79, 63	Precipitate

A summary of these attempts to crystallize purified tRNAs in the ethanol system in the presence of cobalt is given in Table III.

Mounting of Crystals for X-Ray Diffraction Studies. Stabilizing $tRNA^{Phe}$ crystals grown in the presence of cobalt in the ethanol–water system for mounting in capillaries for X-ray diffraction was a major difficulty to surmount in this study. When attempts were made to mount these crystals from the mother liquor, they proved to be difficult to manipulate and easily melted or lost their points and edges, producing a somewhat rounded crystal on which faces could not be located for alignment in the X-ray beam. Eventually, conditions were devised in which crystals tolerated short exposures to atmospheric conditions and still gave good X-ray diffraction patterns after mounting. The mounting solution used for the space group determination and for determination of unit cell dimensions was the same as that in which the crystals were grown except that the 95% ethanol volume fraction was increased 1% over the original and the concentration of magnesium chloride was increased to 0.045 M. An increase in concentration of magnesium chloride to 0.022 M with the same increase in ethanol also allows crystals to be mounted for useful X-ray diffraction studies, or the crystals can be stabilized using only the 1% increase in ethanol concentration while all other solution components are kept constant.

In mounting $tRNA^{Phe}$ crystals for X-ray diffraction studies, precautions must be taken. When a slide was to be mounted, it was quickly removed from the chamber and placed under the dissecting microscope. Approximately 0.1 ml of mounting solution was added, whereupon a slight precipitate formed. This was withdrawn, being careful not to disturb the crystals, by using a short segment of 6-mm i.d. glass tubing which had been drawn to form a fine diameter tip (0.02 mm or less). Mounting solution was again added, and the washing operation was repeated until the slide was free of all precipitate and debris except the crystals. The crystal to be mounted was then selected and freed from the bottom of the slide by moving it carefully with a thin glass fiber. The crystal remained stable when left in this solution for several weeks if the slide was returned to the original crystallization chamber.

The crystal was mounted by drawing it, with solution, a distance of about 1 cm into a 0.7-mm quartz capillary.[3] A filter paper strip (Whatman No. 3MM) was then inserted into the liquid column from the large end of the capillary, and the liquid column containing the crystal was allowed to move into the filter paper by capillary action. After the liquid column had moved about 1 cm, a short column of mounting solution (about 0.5 mm long) was inserted into this vacancy and the small end of the capillary was then sealed with vacuum grease. The crystal was now in the meniscus of the original liquid column and had a liquid column of mounting solution both above and below it with an air space about 0.5 mm long between. (These latter operations must be done quickly, since the solutions containing the crystal are exposed momentarily to the atmosphere at several stages in the procedure.) The remainder of the original mounting solution was now removed with filter paper strips inserted from the large end of the capillary. The crystal was then dried, and final alignment was made in the tube by carefully nudging the crystal with filter paper strips. After alignment had been done, another 0.5-cm liquid column of mounting solution was added within 0.5 cm of the crystal from the large end of the capillary. Both ends were then sealed carefully with vacuum grease, and the crystal was then ready for X-ray diffraction studies. Crystals mounted in this manner have remained stable for 2 weeks at 8–10° for up to 100-hr exposure in the X-ray beam.

Evidence That Crystals Are tRNA. Determination of the ultraviolet absorbancy produced by dissolving an isolated crystal of measured dimensions demonstrated that crystals of both $tRNA^{fMet}$ and $tRNA^{Phe}$ were RNA. At no time were crystals observed in samples containing mixed transfer RNAs or in control solutions without RNA, and a sample of 100% pure *E. coli* $tRNA^{Phe}$ yielded crystals of identical form with those obtained from 65% pure *E. coli* $tRNA^{Phe}$.

When $tRNA^{Phe}$ crystals were isolated from the mother liquor and redissolved, a large increase in amino acid acceptor was noted for the crystals over both the original $tRNA^{Phe}$ solution and the mother liquor remaining after the crystals had formed. Redissolved $tRNA^{Phe}$ crystals were shown to be 70% pure, whereas the remaining mother liquor was 50% pure and the starting sample was 60% pure based on amino acid acceptor assays.[4] This indicated that the crystals were $tRNA^{Phe}$. In general, after crystallization of tRNA had occurred, about 50% of the absorbancy at 260 nm remained in the mother liquor.

Amino acid acceptor assays showed 100% retention of acceptor activity for $tRNA^{fMet}$ after the crystals were redissolved. Very little absorbancy at 260 nm remained in the mother liquor after $tRNA^{fMet}$ had crystallized.

X-Ray Diffraction Studies. These crystals have been found useful for preliminary studies of tRNA structure. The X-ray data to date have given diffraction maxima to 10-Å resolution. Figure 6 shows a three-degree precession photograph about the *c* axis of a $tRNA^{Phe}$ crystal. Calculations were made

[3] Obtained from Unimex Caine Co., Chicago, Ill.

[4] These amino acid acceptor assays were done by Dr. U. L. RajBhandary of the Institute for Enzyme Research at the University of Wisconsin.

from this and three-degree precession photographs about the *a* axis.[5] Measurements revealed a hexagonal system with unit cell dimensions $a = 124$ Å and $c = 100$ Å. The space group is $P6_222$ or possibly $P6_2$ (or their enantiomorphs). Kim and Rich (1968) also found a hexagonal system in heavily hydrated crystals of *E. coli* $tRNA^{fMet}$ grown from an aqueous chloroform system. The unit cell they obtained was much larger with $a = 170$ Å and $c = 234$ Å.

Density measurements by floatation of crystals in cyclohexane–carbon tetrachloride solution determined the crystal density to be 1.36 ± 0.02 g per ml. By using a $\bar{v} = 0.529$ (Henley *et al.*, 1966) for mixed yeast tRNA in solution at 10°, calculations revealed 24 molecules/unit cell. Further details on the results of X-ray diffraction studies with these crystals have been given by Connors and Labanauskas (1969).

Discussion

Simple reproducible conditions have been developed for the crystallization of tRNA. Identical procedures, except for initial tRNA concentration, yielded single crystals from both *E. coli* $tRNA^{Phe}$ and yeast formylmethionine tRNA. This would seem to indicate that these conditions may well be applicable to many pure tRNA species. Preliminary attempts to use this condition for crystallization of seven other purified tRNAs from both yeast and *E. coli* have not been successful. Attempts to crystallize 10 partially purified tRNAs from yeast using this same ethanol–water system have also been unsuccessful. However, Fresco *et al.* (1968) have been able to crystallize unfractionated tRNA using three different systems.

Although these crystals must be kept at temperatures below about 10° and handled in vapor of controlled ethanol concentration, the manipulations needed for X-ray crystallography have been successfully carried out on one class of crystals. X-ray crystallographic studies have been initiated, and a successful determination of the space grouping and unit cell dimensions has been carried out. These crystals give diffraction maxima to 10-Å resolution, which allows studies to be made of molecular conformation. This resolution, however, is not nearly good enough for the fine structure determination that has been done for several proteins, which give good diffraction maxima to 1.5 Å.

By varying tRNA concentration, heavy metal type and concentration, and other solvent parameters, several different crystalline growth patterns have been obtained. Small changes of these variables have led to several improvements in crystal quality and give promise that further modifications will produce crystals with the properties necessary for the determination of the three-dimensional structure of tRNA.

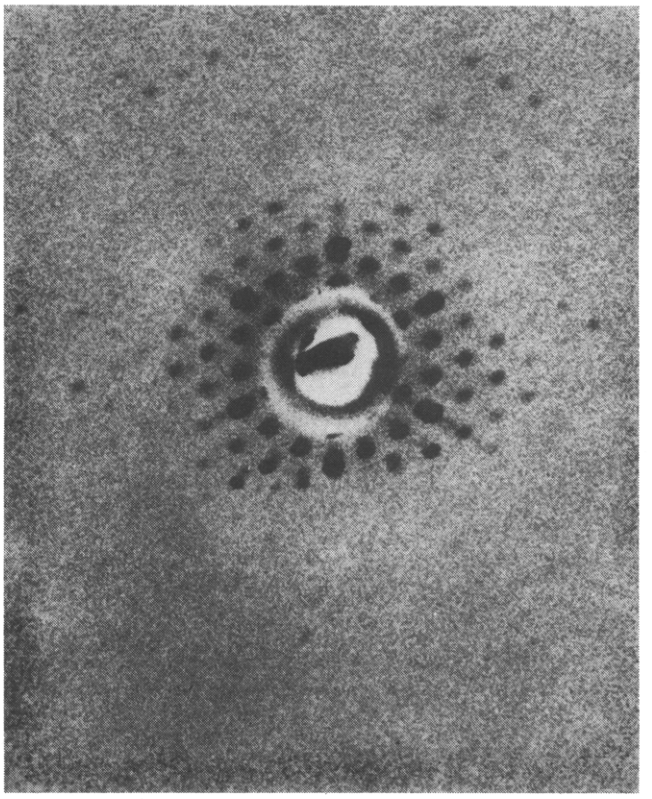

FIGURE 6: A three-degree X-ray precession photograph taken about the unique crystal axis of a $tRNA^{Phe}$ crystal.

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[5] These diffraction patterns were taken and calculations made by Peter G. Connors and Mindaugas Labanauskas (Connors and Labanauskas, 1969) in the Laboratory of Biophysics at the University of Wisconsin, Madison, Wisconsin. Photographs were taken on a Buerger precession camera with 40-kV, collimated, unfiltered copper radiation from a Jarrell Ash microfocus X-ray unit. Work was done in a cold room at 8–10°.

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Amino Acid Differences between Highly Cross-Reactive Antibodies*

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ABSTRACT: Antibodies to *p*-azophenylarsonate and its closest chemical analog, *p*-azophenylphosphonate, were shown to be highly cross-reactive. In competitive binding experiments with either antibody, the heterologous hapten was almost as effective as the homologous hapten in displacing the reference ligand. The similarity in binding properties was paralleled by the similarity in the amino acid compositions of the two antibodies. The amino acid contents of the respective heavy chains differed by a single tyrosine residue, while the contents of the light chains were indistinguishable. These results with highly cross-reactive antibodies provided further support that the multiple amino acid differences previously observed for noncross-reactive antibodies are related to their immunological reactivity.

Characteristic differences in amino acid composition have been found for five rabbit antibodies, anti-phenylarsonate, anti-phenylsulfonate, anti-benzoate, anti-phenyltrimethylammonium, and anti-phenyl β-lactoside (reviewed in Koshland, 1966). These compositional differences were shown to be independent of the charge on the antigenic determinant, the simultaneous production of other antibodies, genetic markers located in the variable sequences of the heavy chain (Koshland, 1967), and the class of immunoglobulin isolated (Koshland *et al.*, 1969). Furthermore, the amino acid differences could not be satisfactorily explained by selective synthesis in subclasses of the variable regions since a separate subclass would be required for each antibody. It appeared, therefore, that the observed differences were associated with the immunological specificities of the respective antibodies.

There was no cross-reaction in ligand binding among the five antibodies studied (Landsteiner, 1945; Kreiter and Pressman, 1964), although three, the anti-phenylarsonate, the anti-phenylsulfonate, and the anti-benzoate, were directed against haptens with similarities in structure and charge. Moreover, the compositional differences were found to be as large among the anti-phenylarsonate, anti-phenylsulfonate, and anti-benzoate antibodies as among the antibodies to the chemically dissimilar haptens. To determine both the extent and kinds of compositional differences associated with related specificities, these studies have been extended in the present work to highly cross-reactive antibodies. Phenylarsonate and its closest chemical analog, phenylphosphonate, were chosen as the antigenic determinants because Kreiter and Pressman (1964) have shown that the phenylphosphonate hapten was a strong inhibitor of the precipitation of anti-phenylarsonate antibody by its homologous antigen, and the phenylarsonate hapten was an equally effective inhibitor in the anti-phosphonate system.

Since it is impossible to separate anti-phenylarsonate antibody from anti-phenylphosphonate antibody, the usual technique of minimizing animal variation by preparing the two antibodies in the same animal could not be utilized. As the best alternative, one set of rabbits was immunized with the azoantigens of phenylarsonate and phenyl β-lactoside, while a comparable set was immunized with the azoantigens of phenylphosphonate and the same phenyl β-lactoside antibody. Thus, the anti-phenyl β-lactoside antibody served as a control for any differences in the two sets of animals.

Methods

Preparation of Haptens. The *p*-nitrophenylphosphonic acid was synthesized according to the method of Doak and Freedman (1951); 0.08 mole of *p*-nitrophenyldiazonium fluoroborate, suspended in 100 ml of ethyl acetate, was allowed to react with 0.08 mole of phosphorous trichloride in the presence of 1.6 g of cuprous bromide catalyst. After the volatile by-products of the reaction were removed by steam distillation, any diphenylphosphinic acid produced was crystallized out by concentrating the distilled reaction mixture to 80 ml and incubating overnight in the cold. The

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