low 20 Å, and this is probably related to the large amount of water and the ease of movement of portions of the molecule which are not restrained by lattice interactions. However, a Fourier analysis of these materials, even at a resolution of 20 Å, would be of great value because it would provide information which might lead us to infer the overall shape of the molecule. The double-stranded regions themselves have a diameter of 20 Å.

Transfer RNA molecules are the first pure nucleic acid molecules to form single crystals. This clearly indicates that these molecules have a definite and regular size and shape, such that they can be organized into a lattice array. The initial crystallization of proteins was of great importance in that it helped to focus the thinking of biochemists on a molecule with a fixed and definite configuration, one which many at the time believed to have a variety of shapes and forms. In a similar way the crystallization of tRNA and the recognition of a definite diffraction pattern leads us to conclude that these molecules also have a definite shape and form and one which is amenable to the same type of physical analysis which has proved so successful with the proteins. Unlike proteins (or most proteins), a unique feature of the tRNA molecule is the fact that it can be readily denatured but readily renatures again. In our investigation, for example, some of these preparations were dried completely and then rehydrated and subsequently crystallized. This behavior, unlike that observed with most proteins, suggests that the molecule has a configuration which is readily reversed once the appropriate hydrating medium is supplied.

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Single Crystals of Transfer RNA from Formylmethionine and Phenylalanine Transfer RNA's

Abstract. *Reproducible conditions have been developed for crystallization of transfer RNA. The conditions may be applicable to many pure transfer RNA species since identical procedures (except for initial transfer RNA concentration) yielded good crystals from both yeast and* Escherichia coli *transfer RNA. These crystals, which must be kept at temperatures below about 10°C and handled in vapor of controlled alcohol concentration, have been studied by x-ray crystallography. The availability of crystals of a nucleic acid opens a route for extending knowledge of the tertiary structure of transfer RNA and its relation to important biological functions.*

Techniques have been developed which permit growth of single crystals of transfer RNA (tRNA). The crystals are of sufficient size, stability, and order that good quality x-ray diffraction patterns were observed. Identical procedures were successfully used in producing crystals of phenylalanine tRNA ($tRNA^{Phe}$) and formylmethionine tRNA ($tRNA^{f\text{-}Met}$). The former was isolated from *Escherichia coli* and the latter from yeast, indicating considerable generality in this procedure for producing the crystals which are essential for determination of three-dimensional molecular structure by x-ray diffraction.

Transfer RNA molecules act as "Rosetta stones" by serving as adapter molecules between the nucleotide sequences of messenger RNA (mRNA) and the amino acid sequences of proteins. An unambiguous translation of mRNA to protein requires that each tRNA interact with an appropriate mRNA codon and also be specifically recognized by the corresponding aminoacyl tRNA synthetase. Genetic experiments have shown that this enzymic recognition does not involve the three anticodon bases in tRNA (*1*). The three-dimensional conformations of tRNA's must share in common the property of binding to ribosomes and being recognized by certain modifying enzymes (*2, 3*). In addition, certain tRNA molecules are recognized by methionyl tRNA formylating enzymes (*4*), methylating enzymes (*5*) and several other enzymes which introduce specific modifications, including the addition of the cell division hormones into tRNA (*6*).

It is known that tRNA folds into a compact structure in solution (*7*). The thermal stability of this structure and the reversible denaturation of biological activity indicate the importance of specific modes of folding of the polynucleotide chain (*8*). Comparison of the primary nucleotide sequence of various tRNA's (*3*) reveals several regions that are capable of forming Watson-Crick base pairs. A secondary structure in accord with these sequences may be an important factor in determining the conformation of tRNA, but an understanding of the basic mechanisms of tRNA function would be greatly enhanced by a detailed knowledge of the three-dimensional structure. By analogy with the recent advances made in the study of proteins (*9*), x-ray crystallographic analyses on single crystals of tRNA are expected to be valuable for this purpose. As the first phase of work in this direction, we report here a method for obtaining single crystals of (*E. coli*) $tRNA^{Phe}$ and (yeast) $tRNA^{f\text{-}Met}$, and conditions necessary for the stabilization of these crystals.

Techniques for purification of tRNA on the milligram to gram scale have been perfected, and several groups of investigators have purified tRNA to a stage approaching a single species (*10*). The Oak Ridge National Laboratories has a program, under sponsorship of the National Institutes of Health, which makes possible the distribution of useful amounts of purified tRNA's from *E. coli* (*11*).

After several pure species of tRNA became available, we began a systematic survey of conditions likely to promote the growth of single crystals.

These conditions were selected to include salt and solvent conditions in which tRNA was known to survive with full retention of biological activity. The first step was to measure solubility of tRNA, with temperature, ethanol concentration, ammonium sulfate concentration, and polyvalent cation concentration being used as the variables. After we determined the ranges of temperature, alcohol, and ammonium sulfate concentration at which precipitation occurred, we assembled a number of chambers in which the vapor composition could be controlled. Droplets containing highly purified tRNA samples were equilibrated with these vapors. In general, the ethanol concentration or ammonium sulfate concentration was adjusted to slowly approach the condition where amorphous precipitation was known to occur. Several days to weeks were allowed for the equilibration. Each of the samples in the chambers was inspected at approximately weekly intervals. If all of the droplets containing the samples were clear, the precipitation variable was intensified by a measured increase in alcohol or ammonium sulfate concentration in a large solvent reservoir within the chamber. If precipitation had occurred, the concentration of precipitating agent was decreased until the precipitate dissolved, and then vapor equilibration was repeated to approach more slowly the precipitation zone or to approach a lesser final concentration of precipitating agent.

After trying a large number of conditions, we found that single crystals could be grown from an ethanol-water medium containing potassium chloride, magnesium ions, and cobalt ions. Both (*E. coli*) $tRNA^{Phe}$ and (yeast) $tRNA^{f\text{-}Met}$ were crystallized in this medium. In addition, alcohol solutions containing magnesium or spermidine also yielded crystals of $tRNA^{Phe}$. Concentrated ammonium sulfate solutions containing added magnesium ions also permitted crystallization of tRNA. We now report the conditions which led to crystallization in the alcohol medium as well as precautions necessary for preserving these crystals while mounting them in capillaries for x-ray crystallographic study.

Phenylalanine tRNA from *Escherichia coli* (B) was supplied by the Oak Ridge National Laboratories (*12*). The purity of this tRNA was approximately 65 percent, as measured by the number of moles of phenylalanine accepted per mole of terminal adenosine in the sample. Purified formylmethionine tRNA from yeast was prepared as described (*13*). All salts were reagent grade except for spermidine which was Aldrich No. 5381. The 95 percent ethanol was obtained from Commercial Solvents Company, Chicago, Illinois.

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 or removal 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 we could make observations with a low power microscope without opening the chamber.

These chambers were constructed by

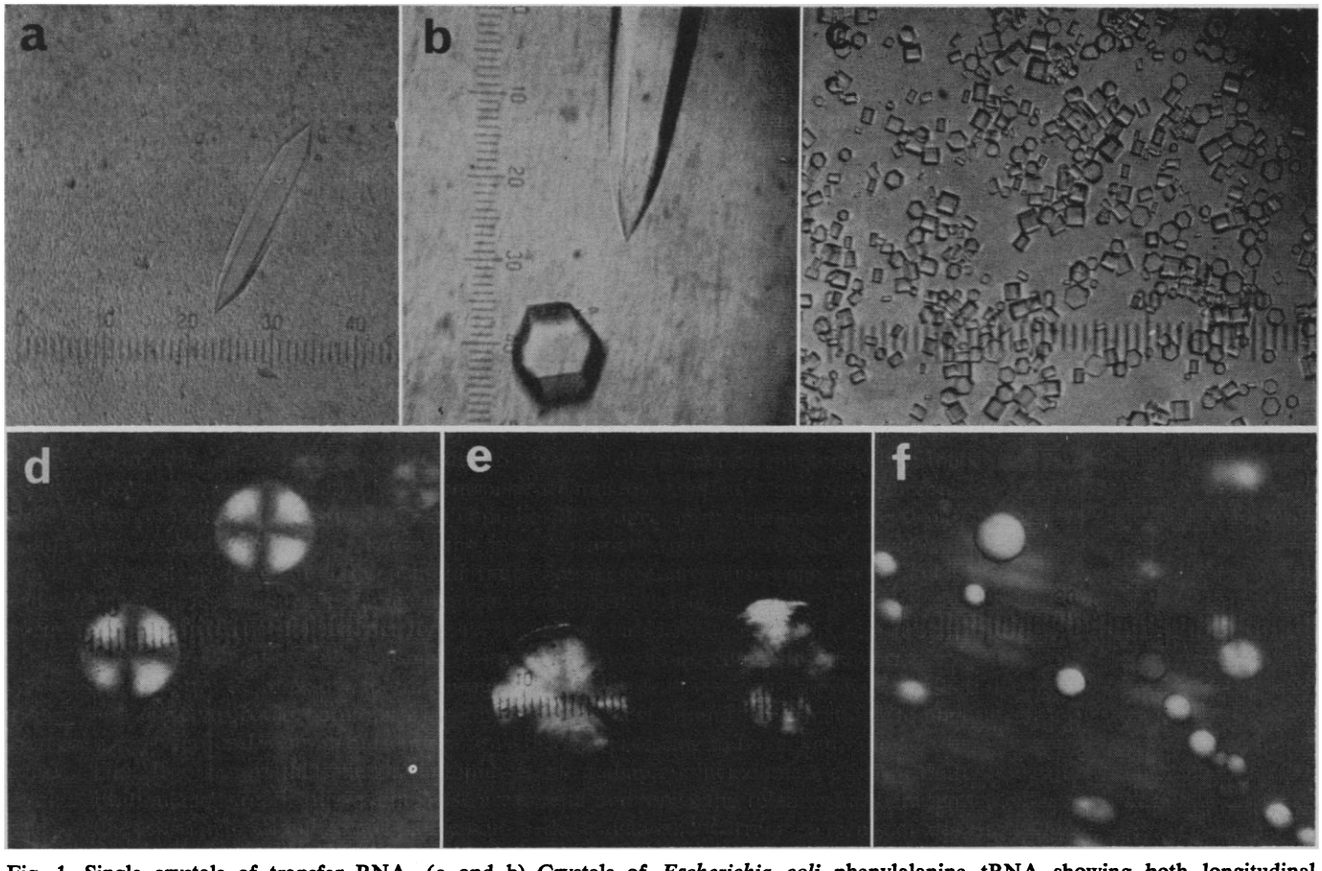

Fig. 1. Single crystals of transfer RNA. (a and b) Crystals of *Escherichia coli* phenylalanine tRNA showing both longitudinal and cross sectional views; (c) yeast formylmethionine tRNA crystals in the form of hexagonal prisms; (d) true spherulites; (e) partially ordered structures; and (f) internally ordered spherulites of phenylalanine tRNA grown from ammonium sulfate solutions. In all photomicrographs, the ruled scale represents 0.02 mm/unit.

grinding flat the upper lip of a Pyrex pie plate (23 cm diameter) and constructing a thick (0.5 cm) 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 an 18-cm disk of lucite supported on legs (1-cm high) of lucite. This allowed the microscope slides to be placed above the large liquid reservoir. Single-cavity slides (7.5 by 2.5 cm) with a cavity 1.75 mm deep and 18 mm in diameter, were obtained from Schaar Scientific Company, Chicago, Illinois. These had been treated with dimethyldichlorosilane 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 the formation of a crust or film of dried buffer at the edge of the droplet. Our experience while growing large numbers of different crystalline samples indicates that the initial concentration of tRNA in the droplet, the concentration of magnesium and cobalt ion, and the final concentration of ethanol are all important variables for the growth of well-formed, large, single crystals.

A typical procedure by which single crystals of either formylmethionine tRNA or phenylalanine tRNA can be grown in alcoholic solutions is as follows: The reservoirs of the crystallization chamber contained a solution composed of 42 ml of water and 11.0 ml of 95 percent ethanol. Into each cavity of one to ten microscope slides in the chamber was pipetted 0.020 ml of a solution of 0.001*M* $CoCl_2$, 0.005*M* $MgCl_2$, 0.05*M* KCl, and 0.005*M* tris adjusted to *p*H 7.4 with HCl. These solutions contained the tRNA samples to be crystallized. For (*E. coli*) $tRNA^{Phe}$ enough RNA was added to give an absorbancy of 98 at 260 nm. A somewhat lower initial absorbancy of 65 to 75 at 260 nm gave good crystals of (yeast) $tRNA^{f\text{-}Met}$. The closed chamber was allowed to equilibrate at 8° to 10°C until crystallization had occurred. This could be as short as 3 days or as long as 2 weeks.

Crystals obtained for (*E. coli*) $tRNA^{Phe}$ were generally about 0.1 by 0.4 mm, although occasionally they grew as large as 0.5 by 2.0 mm. They were always hexagonal in cross section with pointed ends (Fig. 1, A and B). Crystals from (yeast) $tRNA^{f\text{-}Met}$ were generally

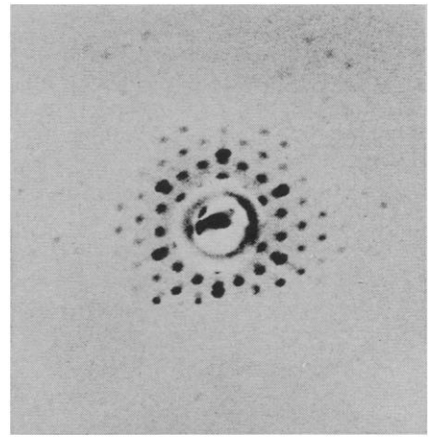

Fig. 2. A three-degree x-ray precession photograph taken about the unique axis of phenylalanine transfer RNA crystal.

smaller hexagonal prisms (Fig. 1C) and exhibited pointed ends only when $CoCl_2$ was not used and the $MgCl_2$ concentration was lowered to 0.001*M*.

These crystals could be stabilized for mounting in capillaries by adding to each drop 0.10 ml of a solution of the same composition as used to grow the crystals, except that the $MgCl_2$ concentration was increased to 0.04*M* and the ethanol was raised to 20.5 percent by volume. If the concentration of magnesium was lower, the crystals were very difficult to manipulate because of their extreme sensitivity to slight changes in solvent composition and to even momentary increases in temperature. A short liquid column of the above solution was placed in each end of the capillary which contained the mounted crystal. The capillaries were sealed with vacuum grease and then used for x-ray diffraction studies.

When the crystals are being mounted in capillaries for x-ray diffraction, it is important to minimize exposure of solutions containing the crystals to the atmosphere. When the aforesaid precautions were followed, crystals were successfully mounted. However, use of a "glove box" containing the same atmosphere as that in which the crystals were grown is recommended for the safe handling of the crystals.

In addition to the procedure described, crystals could be grown in solvents where the $CoCl_2$ was replaced with 0.001*M* spermidine and in solvents containing no polyvalent salts at all with all other solution components unchanged. Crystals were also grown from solutions of the same mixture essentially with 0.001*M* $MgCl_2$ as the only polyvalent cation. However, these crystals were much smaller than those obtained in the presence of $CoCl_2$, and successful crystallization was less frequent. Only the crystals grown in cobalt-magnesium-alcohol have been studied by x-ray diffraction.

Determination of the ultraviolet absorbancy produced by dissolving an isolated crystal of measured dimensions demonstrated that the crystals were RNA. At no time were crystals observed in samples containing mixed transfer RNA's or in control solutions without RNA. A sample of 100 percent pure (*E. coli*) $tRNA^{Phe}$ yielded crystals of identical form to those obtained from 65 percent pure (*E. coli*) $tRNA^{Phe}$. Both $tRNA^{Phe}$ and $tRNA^{f\text{-}Met}$ crystals retained their amino acid acceptor activity when redissolved.

Spherulites were obtained when attempts were made to crystallize tRNA from ammonium sulfate solutions. The photographs in Fig. 1, D–F, were taken through crossed polaroids. The differences in birefringence indicate differences in order within the crystals. Figure 1D shows true spherulites with the characteristic extinction cross (*14*). 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 *p*H 7.4, with an ammonium sulfate concentration that varied from 30 to 58 percent saturation. The ammonium sulfate solutions were saturated at 5°C, and crystal growth was at 8.5°C.

The material shown in Fig. 1E has regions of crystalline orientation, and Fig. 1F shows material which is ordered internally to give a single angle of rotation. These samples were grown in the same way as those in Fig. 1D, except the $CoCl_2$ was replaced by $CuCl_2$, and the ammonium sulfate concentration was varied from 30 to 53 percent. Because of their spherical habit of growth, these crystals have not yet proven useful for single-crystal x-ray diffraction studies (*15*).

Figure 2 shows a three-degree precession photograph about the unique axis of a $tRNA^{Phe}$ crystal. The space group is $P6_222$ or possibly $P6_2$ (or their enantiomorphs). The hexagonal unit cell has dimensions $a = 124$ Å, $c = 100$ Å, and contains 24 molecules of tRNA. The volume fraction of tRNA is 0.42, similar to many protein crystals (*9*). Still photographs show clearly discernible diffraction maxima to 10 Å resolution. Photographs were taken on a Buerger

precession camera with 40 kilovolt, collimated, unfiltered copper radiation from a Jarrell-Ash microfocus x-ray unit. All work was done in a cold room at 8° to 10° C. Crystals still gave clear diffraction patterns after 100 hours exposure in the x-ray beam.

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Nondependence of Frequency on Mass: A Differential Experiment

Abstract. *A differential Loran C experiment, which is independent of variations in clock rates, shows that the frequency of a radio transmission is not affected by the mass of the earth.*

Sadeh *et al.* have reported that the frequency of a radio transmission propagated along the surface of the earth decreases by 2 parts in 10^{12} every 1000 km (*1*). The frequency of the Loran C radio transmission from Cape Fear, North Carolina, controlled by a cesium-beam atomic clock, was compared to the frequency of a similar clock mounted in a truck at distances up to 1500 km away; the frequency was found to decrease with distance. This change in received frequency, which is here called the "horizontal effect," was attributed to the mass of the earth. It is not the same as the gravitational red shift of relativity, which relates the difference in frequency of two atomic clocks at different potential heights.

Announcement of the horizontal effect (*1*) was puzzling because it is contrary to the theory of relativity. Therefore, additional experiments have been conducted at Marquette University. In the first set, completed 6 September 1968, the frequencies of transmissions received from Loran C stations and from U.S. Naval radio stations as far away as Northwest Cape, Australia (17,000 km), were compared with the cesium-beam atomic clock at Marquette. These transmissions are controlled in time and frequency by the U.S. Naval Observatory, where calibration of the atomic clocks at the transmitters and the clock at Marquette was done. However, no change in frequency was found within the errors of observation at Marquette for any station (*2*). For Northwest Cape, the computed decrease based on the horizontal effect is 3.4 parts in 10^{11}, or one order larger than the maximum change observed at the Naval Research Laboratory (*1*).

Results from both NRL and Marquette would be affected by changes in the rates of the atomic clocks at either the transmitting or observing stations. However, we can eliminate the effects of changes in clock rates by performing a differential Loran C experiment. Loran C is a pulsed, hyperbolic, radio navigational system, which operates on 100 khz. The stations of a chain are synchronized through the continual interchange of pulses (*3*). The pulses emitted at each station are locked to the frequency of the carrier wave. Hence, the transmitted frequencies are the same for all stations of a chain. Measurement of the difference in arrival times of selected cycles of pulses from day to day gives the difference in frequencies of the carrier waves as received. The difference is independent of variations in the rates of any clocks

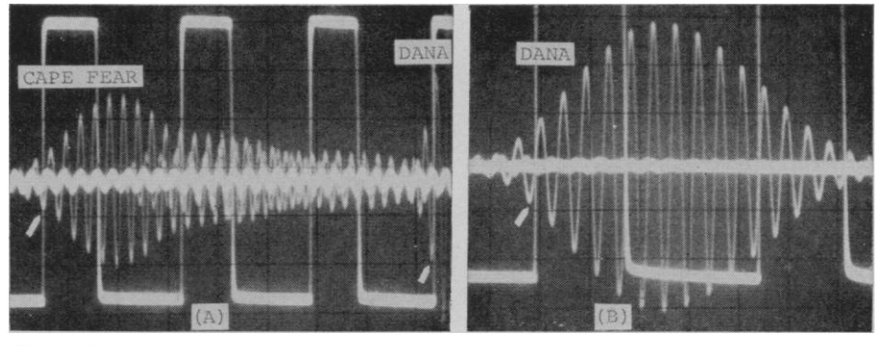

Fig. 1. Loran C pulses from Cape Fear, North Carolina, and Dana, Indiana, received at Marquette 19 October 1968 at 1530 U.T. (universal time) and time markers. Periods are 10 μsec for Loran C cycles and 100 μsec for markers. Cycles measured are marked with arrows. (A) When a minimum from Cape Fear coincided with a time marker, the minimum from Dana was 2.4 μsec ahead of the nearby marker. (B) The pulse from Dana has been expanded horizontally with the amplitude reduced for measurement. Coincidence setting on the pulse from Dana was made after photographs were taken. Early cycles are measured to ensure reception of the ground wave. Sky waves, which have slightly variable transmission times, arrive after the ground wave.