Effect of Cyanoethylation of Yeast Transfer Ribonucleic Acid on Its Amino Acid Acceptor Activity*

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ABSTRACT: Yeast transfer ribonucleic acid (t-RNA) has been cyanoethylated with acrylonitrile at 37° in dimethyl sulfoxide containing 1 M dimethylaminoethanol and varying amounts of water. The water content controlled the number of pseudouridylate residues (ψp) cyanoethylated. In 10% water only about 0.3 ψp residue were reacted/t-RNA molecule; in 25% water, 1.2 residues; in 35% water, 2 residues; and in 50% water, all the ψp reacted (3 per t-RNA molecule). All reactions but the first were complete in 90 min and little apparent cyanoethylation occurred after this time. The greater the degree of cyanoethylation of ψp the greater the loss of secondary structure as shown by the melting curve of the t-RNA, by the change in its optical rotatory dispersion, and by the reduction in its amino acid acceptor activity. Cyanoethylation of all the ψp led to a loss of about 90% of the acceptor activity. It had no effect on the incorporation of nucleotides into the terminal pCpA sequence. The data indicate that all t-RNA's in the mixture contain in common a specific ψp which is exposed for reaction in 25% water and this ψp is important in facilitating the attachment of amino acids.

Amino acid transfer ribonucleic acids (t-RNA's) are unique among the families of polynucleotides in that they have a coenzyme-like function in accepting specific amino acids in an activated state from one enzyme system and correctly delivering them to another, the protein-synthesizing polysome complex (Brown, 1963). They are also unique in possessing a number of "odd" nucleotides including ψp, inosinic, dihydrouridylic, and thiouridylic acids, and a variety of methylated nucleotides. The function of these "odd" nucleotides in t-RNA is unknown. Some may function specifically, for example, by separating the anticodon from the rest of the polynucleotide chain thus preventing errors in reading (Holley *et al.*, 1965). Others may function less specifically by being involved in the maintenance of structure of the t-RNA. This structure may or may not be of critical importance. A third possibility, which may be related to the second, is that some of the "odd" nucleotides have no function in the reactions of t-RNA but rather protect it from cellular degradation.

One approach for studying the function of these nucleotides is to modify them by reaction with a specific reagent and to note the effects of this modification on physical and biological properties of the t-RNA. Acrylonitrile was found by Chambers (1965) to react under controlled conditions almost specifically with ψ. This observation has been exploited by Yoshida and Ukita (1965a) and Ofengand (1965) to block the ψp residues of t-RNA. They compared the differences in reaction rate of ψp and the other nucleotides of t-RNA under different conditions of pH and salt concentration. In particular Yoshida and Ukita (1965b) noted that the reaction of acrylonitrile with the ψp in t-RNA in aqueous medium was a function of the sodium chloride concentration. In the presence of 0.5 M sodium chloride a sharp break in the reaction curve was noted after only one ψp had reacted.

We have reacted acrylonitrile with the ψp of t-RNA in a DMSO–water system which allowed us to control the number of these residues cyanoethylated. We have concluded from physical and biological studies that the ψp is involved in maintaining the secondary structure of the t-RNA and that one of the ψp residues, which is common to all t-RNA chains in yeast, is intimately associated with amino acid acceptance by the t-RNA. However, since complete cyanoethylation of all ψp in t-RNA did not lead to complete loss of acceptor activity this particular residue is not absolutely essential for activity.

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[1] Abbreviations used: dimethyl sulfoxide, DMSO; trichloroacetic acid, TCA; pseudouridine, ψ; pseudouridylic acid or a pseudouridylic acid residue, ψp; RNA that has been reacted for various periods of time in various DMSO–water solutions will also be designated in the following way: 35_{180} C-t-RNA refers to commercial t-RNA reacted for 180 min in 35% water, 65% DMSO solvent. The control samples without acrylonitrile are designated as $B10_{90}$ L-t-RNA which refers to a control sample, without acrylonitrile, of t-RNA prepared in this laboratory reacted for 90 min in 10% water–90% DMSO.

Materials and Methods

Materials. DMSO and dimethylaminoethanol were vacuum distilled twice. Acrylonitrile (Eastman, practical grade) was used without purification. Radioactive compounds were from New England Nuclear Corp. and enzymes from Worthington Biochemical Corp., unless otherwise stated. Radioactivity was determined in a Nuclear-Chicago D47 windowless, gas-flow planchet counter or a Nuclear Chicago Unilux scintillation counter using Bray's (1960) solution. Melting curves of t-RNA were determined in a Gilford 2000 spectrophotometer and optical rotatory dispersion in a Jasco ORD/UV-5 spectropolarimeter. Absorption spectra were determined on a Cary 11 spectrophotometer. Optical density refers to that measured at 260 mμ in a 1-cm cell unless otherwise specified.

Preparation of t-RNA. Commercial yeast t-RNA (Calbiochem Lot No. 40278) and t-RNA prepared in this laboratory from baker's yeast (Bell *et al.*, 1964) were passed through a column (25 × 2 cm) of Dowex 50-X4 (500–100 mesh) in the dimethylhydroxyethylammonium form. The eluted RNA was almost free of Na^+ as determined by a flame test. The eluate was lyophilized.

Properties of t-RNA. t-RNA (5 mg) in the dimethylhydroxyethylammonium form was completely soluble in 2 ml of 100% DMSO and nearly soluble in 1 ml. The optical densities at 260 mμ of these t-RNA's in distilled water were 15.1/mg for commercial t-RNA (C-t-RNA) and 11.8/mg for laboratory *t*-RNA (L-t-RNA). Using the King (1932) method for phosphate determination 1 μmole of phosphate was equivalent to 7.7 ODU of t-RNA. On the basis of 80 nucleotides/t-RNA molecule, 10 ODU were equivalent to 16.3 mμmoles of t-RNA. The proportion of terminal adenosine of the C- and L-t-RNA was determined as described earlier (Bell *et al.*, 1964). C-t-RNA was found to have 33.5% of the molecules ending in adenosine while L-t-RNA had 75.5%. The rest of the molecules ended in cytidine.

Conditions of Reaction. Cyanoethylation was carried out in a DMSO–water solution made 1.0 M with dimethylaminoethanol. The RNA or nucleosides were added to the level of 5.0 mg/ml. The proportion of water was varied. The majority of the reactions were performed in 10–50% water. To each 1 ml of solution was added 0.03 ml (0.45 mmole) of acrylonitrile. The solutions were incubated at 37° for various periods of time, usually for 60, 90, and 180 min. Samples without acrylonitrile were incubated for comparable periods as controls.

Collection of Reacted Materials. I. CYANOETHYLATED t-RNA. (a) The RNA samples for biological and physical studies after reaction for various periods of time were neutralized with glacial acetic acid (0.05 ml/ml of reaction solution). A few drops of 5 M sodium chloride were added and finally the RNA was precipitated with four volumes of ice-cold 95% ethanol. The precipitate was collected by centrifugation and washed twice with ether. The pellet was air dried and dissolved in distilled water (1 ml/ml of reaction solution). (b) RNA cyanoethylated with [^{14}C]acrylonitrile was handled differently. At intervals 0.1-ml aliquots of the solution were put into 1.0 ml of ice-cold 10% TCA. After 1 hr the precipitate was collected on premoistened 2.5-cm glass fiber filters and washed with 80 ml of ice-cold 10% TCA (Britten *et al.*, 1955). The pads were dried and their radioactivity was determined. The quantity of acrylonitrile which reacted/80 mμmoles of RNA phosphate (approximately 1 mμmole of t-RNA) was calculated from the specific activity of the acrylonitrile (3600 cpm/mμmole) and the optical density of the RNA.

II. CYANOETHYLATED NUCLEOSIDES. (a) The reacted nucleosides were collected by spotting some of the reaction solution on Whatman No. 1 paper at specific time intervals. The paper was developed by descending chromatography using the isopropyl alcohol–water–ammonia system (7:2:1, v/v). After development, the nucleosides were eluted from the paper and their absorption spectra were determined. (b) Cyanoethylation of ψ caused a shift in the wavelength of maximal absorption in alkaline solution from 286 to 266 mμ. Thus it was possible to follow the reaction of ψ by following the loss of optical density at 286 mμ. Alternatively the change in absorption at 300 mμ can be used since DMSO does not absorb at this wavelength and cyanoethylated ψ has negligible absorption at the levels used here. Aliquots (0.01 ml) of the reaction solution were taken at various times and put in 1.0 ml of distilled water. The dimethylaminoethanol in this aliquot was sufficient to make the solution strongly basic. The loss of optical density at 300 mμ was found to agree with the formation of cyanoethylated ψ as determined by paper chromatography.

Products of Cyanoethylation of t-RNA. The products of cyanoethylation of t-RNA were separated on a column of Dowex 1 (formate) resin. C-t-RNA (100 mg) was digested with 920 μg of Takadiastase ribonuclease T_2 (Rushizky and Sober, 1963) for 3 days at 37°, pH 4.5. The pH was adjusted to 6.7 and the digestion was continued for another 24 hr at 37° with 1 mg of pancreatic ribonuclease (RNAase) and 5 units of spleen phosphodiesterase. The digest was then applied to a column (100 × 1 cm) of Dowex 1-X8 resin (200–400 mesh) in the formate form and chromatographed by the method of Cohn (1950) with slight modification. Individual peaks were adsorbed on charcoal, washed to remove residual salt, eluted with 5% ammonia, evaporated to dryness, taken up in water, titrated to pH 8.0, and 1 unit of *Escherichia coli* phosphomonoesterase was added. After 24 hr at 37° the material was chromatographed on paper and the spots (located under ultraviolet light) were identified and measured. The recovery of the various nucleotides agreed well with other estimates by this laboratory (Bell *et al.*, 1964).

In one case the recovered peaks of a digest of 50_{180} C-t-RNA were desalted on a column of Dowex 50 resin (hydrogen form). The eluted nucleotides were evaporated to dryness and then digested with phosphomonoesterase as before.

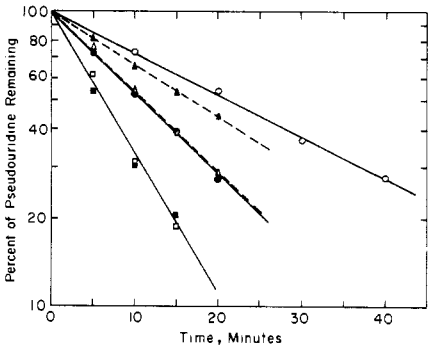

FIGURE 1: Cyanoethylation of pseudouridine. The solutions of 5 mg of pseudouridine/ml in DMSO were 1 M in dimethylaminoethanol and 440 mM in acrylonitrile and contained: no water (○—○), 5% water (●—●), 10% water (■—■), or 50% water (□—□). Additional reactions in 50% water with 0.5 M dimethylaminoethanol–440 mM acrylonitrile (△—△) and 1.0 M dimethylaminoethanol–159 mM acrylonitrile (▲—▲) are shown. The solutions were incubated at 37°. The cyanoethylation of pseudouridine was followed by the loss of optical density at 300 mμ in an alkaline solution. At the indicated times 0.01 ml was taken into 1.0 ml of water and the optical density was read at 300 mμ in a cell of 1-cm light path. There was sufficient dimethylaminoethanol in the sample to make the water alkaline.

Chromatography of t-RNA on a Column of Benzoylated DEAE-Cellulose. RNA was applied to a column of benzoylated DEAE-cellulose (Tener *et al.*, 1966) (100 × 1 cm), which had been equilibrated with 10^{-2} M magnesium chloride–0.4 M sodium chloride. The column was developed with a 1-l. linear gradient of sodium chloride from 0.4 to 1.0 M and 10^{-2} M in magnesium chloride. When the gradient was exhausted remaining RNA was eluted with a solution containing 10^{-2} M magnesium chloride, 1.0 M sodium chloride, and 10% methoxyethanol. The flow rate was about 40 ml/hr and fractions of 5 ml were collected.

Physical Properties of t-RNA. Optical rotatory dispersion was determined at room temperature in 1.25 mM Tris, pH 7.4–1.25 mM EDTA–1.5 mM magnesium chloride (effective Mg^{2+} concentration 0.25 mM) solution in a cell of 1-cm light path. Sufficient t-RNA was added to give about 2.0 ODU/ml. Specific rotations [α] were calculated for wavelengths from 220 to 300 mμ at 5-mμ intervals. The change in [α] from the minimum in the 240-mμ region to the maximum in the 280-mμ region, Δ[α] min − max, was calculated.

The effects of cyanoethylation were also studied by examining the change in optical density at 260 mμ upon heating the RNA. These melting curves were determined in 5 mM Tris, pH 7.4–5 mM EDTA–6 mM magnesium chloride (effective Mg^{2+} concentration 1 mM). The RNA was added to give an initial optical density of about 0.5–0.6 unit. The samples were measured against a blank of uridylic acid containing 0.5 ODU, in the same buffer. All samples were degassed by brief vacuum treatment before use.

Preparation of Enzyme. Commercial baker's yeast (20 g) was grown overnight at 33° in a medium consisting of 128 g of glucose, 32 g of Difco yeast extract, 24 g of $(NH_4)_2HPO_4$, and 0.8 g of magnesium acetate in 1600 ml of water adjusted to pH 3.5 with lactic acid. The culture was aerated and stirred. The cells were collected by centrifugation, washed twice in 0.05 M Tris, pH 7.4–0.005 M magnesium acetate, and the pellet was divided into four parts. To each part was added 40 g of clean Superbrite glass beads (type 110–5005) (Minnesota Mining and Manufacturing Co., Minneapolis), 0.5 ml of 5% 2-mercaptoethanol, and 10 ml of Tris–Mg^{2+} buffer. They were ground in a Virtis homogenizer at the highest speed for 5 min followed by 3 min of cooling in ice. The cycle of grinding and cooling was repeated three times. After removing the glass beads and cell debris by low-speed centrifugation the supernatant was centrifuged in a Spinco Model L No. 40 rotor at 39,000 rpm for 120 min. The middle three-fourths of the supernatant was collected, diluted to about 80 ml with buffer, dialyzed overnight against 3 l. of buffer with 1 ml of 5% 2-mercaptoethanol, and then stored in liquid nitrogen.

Amino Acid Acceptor Activity. Amino acid incorporation was assayed in 0.23 ml of a solution containing 1.13 μmoles of ATP, 0.23 μmole of amino acids, 0.3 μmole of CTP, 0.8 μmole of EDTA, 3.0 μmoles of magnesium chloride, 0.5 μmole of potassium chloride, and 80 μmoles of Tris, pH 7.0 or 8.0. To this were added 0.05 ml of enzyme and 0.05, 0.1, or 0.15 ml of t-RNA solution. The optical densities of the t-RNA solutions were previously determined in 5 mM Tris, pH 7.4–5 mM EDTA. The molar quantities of t-RNA (about 10 mμmoles for the cyanoethylated RNA assay) added to the assay were calculated from this optical density. All assays were run in duplicate and gave results which varied no more than 7% from the average. The incubations were at room temperature and were terminated, usually after 1 hr, by the addition of 0.5 ml of 5 M sodium chloride and 2.0 ml of ice-cold 95% ethanol. After 1 hr at −10° the precipitates were collected on moistened 2.5-cm glass fiber filters (Britten *et al.*, 1955). These were washed twice with ice-cold 10% TCA containing 1.0 M sodium chloride and twice with ice-cold 10% TCA (total volume of wash 80 ml). The filter pads were dried at 70° overnight to remove TCA and assayed for radioactivity. The results have been corrected for blank samples containing enzyme but no t-RNA.

Results

Kinetics of Cyanoethylation of Nucleosides. The products of cyanoethylation of nucleosides were identified and found to be similar to those reported by Chambers (1965), Ofengand (1965), and Yoshida

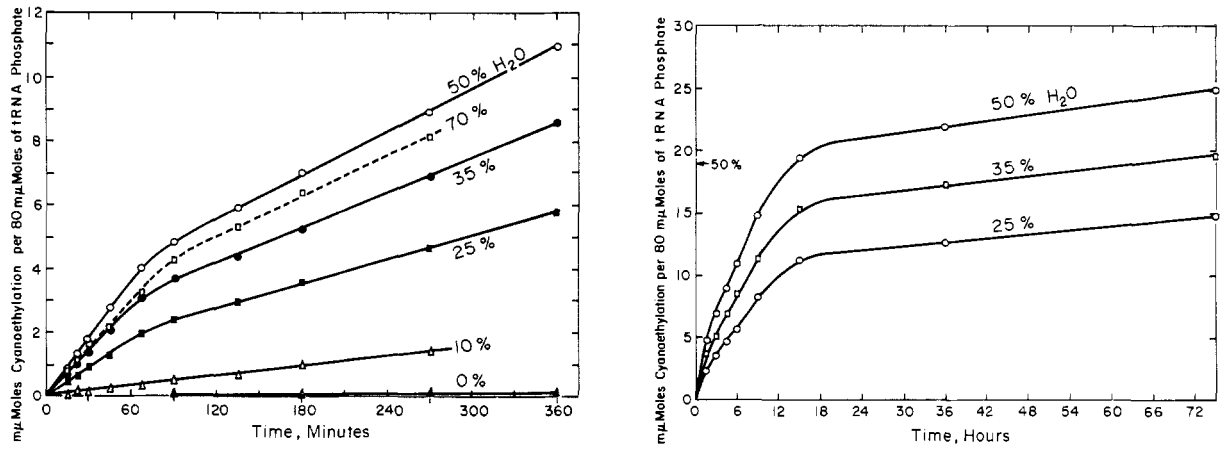

FIGURE 2: Cyanoethylation of t-RNA. The cyanoethylation of t-RNA was followed by the incorporation of [^{14}C]-acrylonitrile, sp act. 3600 cpm/μmole, into the t-RNA at 37°. There were 5 mg of t-RNA/ml of solution. The water content of the solution was varied from 0 to 70% as indicated. In all cases the solutions were 1.0 M in dimethylaminoethanol and 440 mM in acrylonitrile. At the indicated time intervals the RNA in 0.1 ml of the reaction solution was precipitated by 1 ml of ice-cold 10% TCA. After drying and determining the ^{14}C, the acrylonitrile incorporated/80 mμmoles of RNA phosphate was calculated. Figure 2a (left) shows the early incorporation; Figure 2b (right) the long-term incorporation.

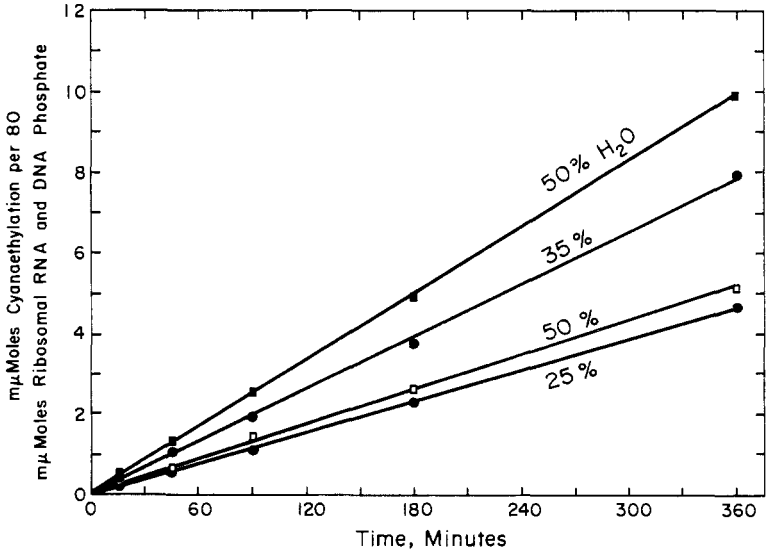

FIGURE 3: Cyanoethylation of ribosomal RNA and single-stranded DNA. Conditions and methods as described in Figure 2. The r-RNA was phenol-treated bovine liver r-RNA that had been purified by three passages through a DEAE-cellulose column with a sodium chloride gradient 0.3–1.0 M. Care was taken to remove any t-RNA after each passage. The DNA was salmon testis DNA isolated by phenol treatment in 0.08 M dimethylhydroxyethylammonium acetate, pH 7.5. The DNA was purified by three precipitations from the dilute buffer with ethanol, the DNA being wound up on a glass rod in the usual manner and denatured by heat treatment in solution. The solid symbols refer to r-RNA and the open symbols to DNA.

and Ukita (1965a). The rates of cyanoethylation determined by loss of optical density at 300 mμ of ψ under various conditions are shown in Figure 1. The rate of reaction was proportional to the concentrations of acrylonitrile and dimethylaminoethanol. Increasing the amount of water in the solution up to 10% increased the rate of reaction. At 10 or 50% water, 50% of the ψ was cyanoethylated in 6 min.

The rate of formation of dicyanoethyl-ψ and cyanoethylation of the other nucleosides were followed by paper chromatography. Uridine, thymidine, and cyanoethylated ψ were 50% cyanoethylated in 1.5

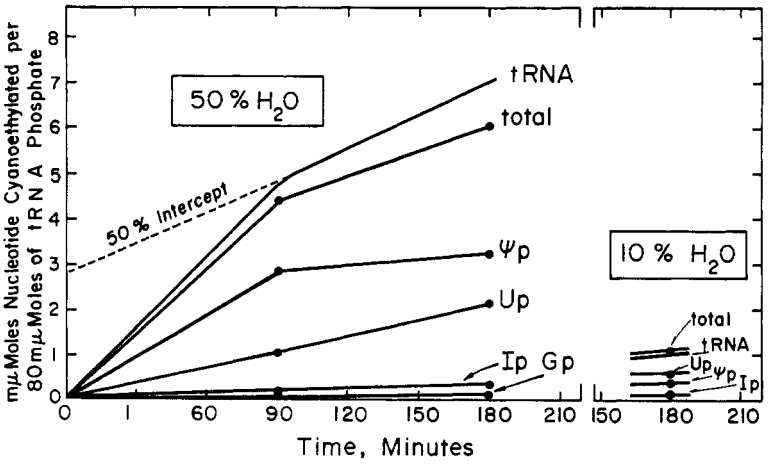

FIGURE 4: Kinetics of cyanoethylation of t-RNA nucleotides. The extent of cyanoethylation of the individual t-RNA nucleotides was determined from a digest of about 100 mg of cyanoethylated t-RNA. The nucleotides were separated on a Dowex 1 column (Figure 5), identified, and measured (as described in Methods). The results for cyanoethylation for 90 and 180 min in 50% water and for 180 min in 10% water are plotted here as mμmoles incorporated/80 mμmoles of t-RNA phosphate. The sum of the reacted nucleotides is also plotted and compared with the cyanoethylation of t-RNA as shown in Figure 2a. The extrapolated intercept due to the initial rapid cyanoethylation of t-RNA in 50% water is also included.

hr in a system of 10% water and 90% DMSO. Guanosine was 50% reacted in 3 hr. Above 10% water these rates were constant.

Kinetics of Cyanoethylation of Nucleic Acids. The cyanoethylation of t-RNA was followed by determining the incorporation of [^{14}C]acrylonitrile into the RNA under various solvent conditions. The results, shown in Figure 2a, should be compared with cyanoethylation of beef liver ribosomal ribonucleic acid (r-RNA) and denatured salmon testis deoxyribonucleic acid (DNA) (Figure 3). Above 10% water the t-RNA had an initial rapid rate of cyanoethylation which lasted for about 90 min and was then followed by a secondary slower rate. The r-RNA showed no initial rapid rate but its primary rate of cyanoethylation was similar to the secondary rate of t-RNA in solutions of the same water content. As will be shown later this rapid initial rate of cyanoethylation of t-RNA was due to cyanoethylation of exposed ψp (see Figure 4). By extrapolation, the initial rapid reaction represents about 3.0 ψp cyanoethylated/80 nucleotides (one t-RNA molecule) in the 50% water solvent; 2.0 residues in 35% water; 1.2 residues in 25% water; and none in 10% water (however, see Figure 4).

The secondary slower reaction of t-RNA was probably due to cyanoethylation of uridylic, ribothymidylic, and inosinic acid residues (Yoshida and Ukita, 1965a) and the second cyanoethylation of ψp (see Figure 4), the total of which should be an average of 22.2 cyanoethylations/t-RNA molecule. The secondary reaction of t-RNA, by extrapolation, at 50% water, represented a further 19 cyanoethyl residues per t-RNA molecule. The primary slow reaction of r-RNA and salmon DNA, also completed in about 15 hr, was due to cyanoethylation of the uridylic and thymidylic acid residues, respectively. These reactions were followed by further cyanoethylation at an even slower rate (see Figure 2b). Finally it should be noted that there was no appreciable cyanoethylation of t-RNA in the absence of water, in contrast to the cyanoethylation of free ψ which was appreciable.

Identification of t-RNA Reaction Products. The nucleotides obtained by enzymic digestion were separated on a Dowex 1 column as shown in Figure 5. This figure also shows the location of cyanoethylated ψp, fraction 110, and cyanoethylated uridylic acid, fraction 129. The results of cyanoethylation for 90 and 180 min at 50% water and for 180 min at 10% water are illustrated in Figure 4.

In 50% water no ψp remained after 180 min while about 10% was unreacted after 90 min. No product of reaction with thymidylic acid could be found. As expected no adenylic or cytidylic acids reacted. The cyanoethylation of inosinic acid was followed by the disappearance of inosinic acid from the peak at fraction 157. From Figure 4, the sum of the cyanoethylated products agreed with the total cyanoethylation of t-RNA as shown in Figure 2a. Thus the initial rapid rise in cyanoethylation of t-RNA was due to cyanoethylation of ψp. The secondary rate of cyanoethylation of t-RNA was due to cyanoethylation of uridylic, guanylic, and inosinic acid residues.

Chromatography of t-RNA on a Column of Benzoylated DEAE-Cellulose. The effect of cyanoethylation of t-RNA upon its pattern of chromatography on a standardized benzoylated DEAE-cellulose column may be seen in Figure 6. About 96% of unreacted RNA was eluted during the salt gradient and only a trace

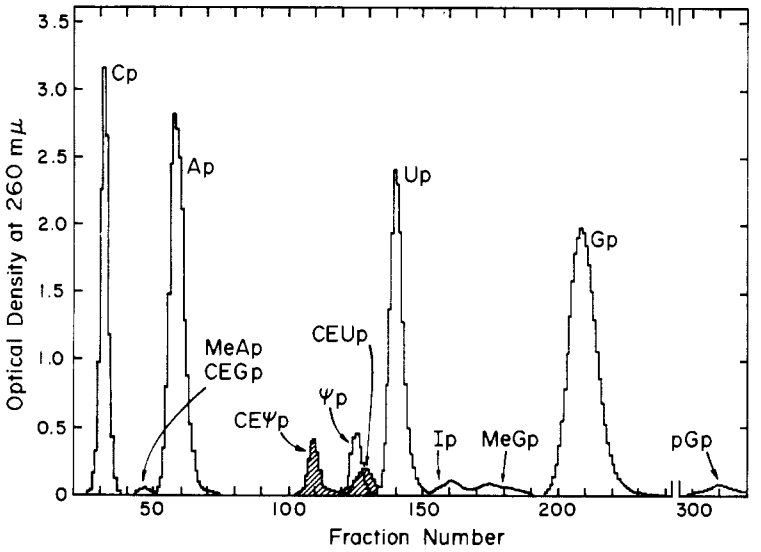

FIGURE 5: Chromatography of nucleotides from a complete enzymic digest of t-RNA on a Dowex 1 column. Approximately 100 mg of t-RNA was digested to nucleotides with Takadiastase ribonuclease T_2 for 3 days at pH 4.5 followed by spleen phosphodiesterase and pancreatic RNAase for 1 day at pH 6.7; all digestions were at 37°. The digest was then washed with water (fractions 1–20) into a 100 × 1 cm column of Dowex 1-X8 resin, (200–400 mesh) in the formate form. The column was eluted at a rate of 230 ml/hr, 10 fractions/hr, and the nucleotides were eluted with the following solutions: 0.1 M formic acid, fractions 21–35; 0.5 M formic acid, fractions 36–65; a linear gradient from water to 1.0 M ammonium formate, 1.0 M formic acid, 4 l. each; fraction 66 to end. The optical density of each fraction was determined at the pH of the eluent (usually below pH 3.0). The nucleotides in the optical density peaks were identified. The pattern of elution of unreacted nucleotides is shown as clear peaks while cyanoethylated pseudouridylic and uridylic acids are shown as shaded peaks. CE refers to a cyanoethylated nucleotide.

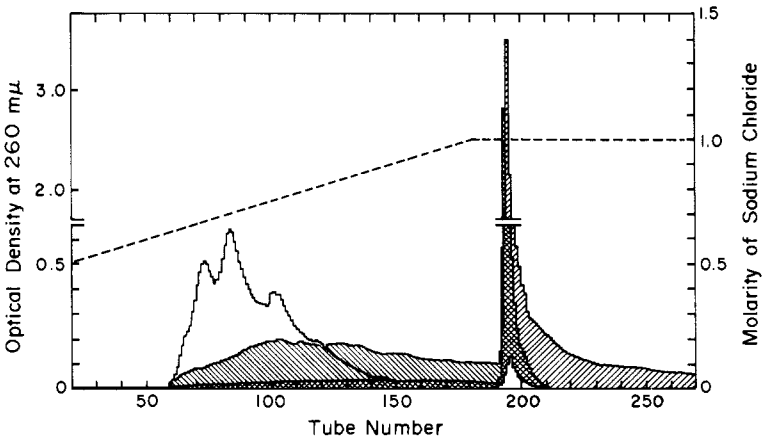

FIGURE 6: Chromatography of t-RNA on a column of benzoylated DEAE-cellulose. Approximately 200 ODU of unreacted t-RNA (clear areas), 25_{180} C-t-RNA (shaded areas), and 50_{180} C-t-RNA (crosshatched areas) were chromatographed individually on a standardized benzoylated DEAE-cellulose column (100 × 1 cm), developed with a linear gradient of sodium chloride from 0.4 to 1.0 M (total volume, 1 l.). Solutions were 10^{-2} M in magnesium chloride. The RNA was applied to the column in the starting salt solution. Fractions were collected at a flow rate of 42 ml/hr. After the gradient had been exhausted the column was eluted with 10^{-2} M magnesium chloride–1.0 sodium chloride–10% methoxyethanol.

was eluted by 10% methoxyethanol–salt (Tener *et al.*, 1966). t-RNA in which the ψp was completely cyanoethylated, 50_{180} C-t-RNA, had no components that appeared during the gradient. All the material that could be eluted (70%) appeared in the 10% methoxyethanol. Thus there were no t-RNA molecules remaining that were similar to normal t-RNA. t-RNA which had about one ψp cyanoethylated per molecule (25_{180} C-t-RNA) also showed a greatly altered pattern. About one-third of the material appeared in the

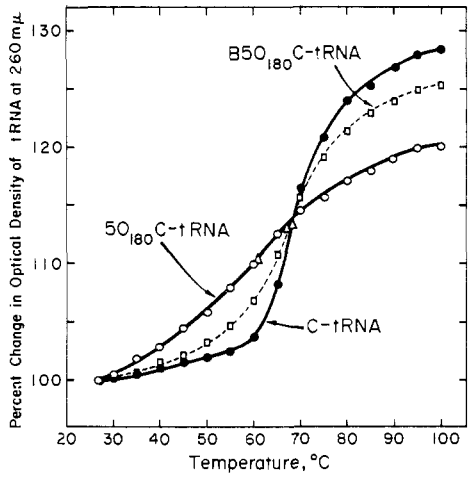

FIGURE 7: Melting curves for unreacted and cyanoethylated t-RNA. The per cent change in optical density at 260 mμ with temperature is plotted for unreacted RNA, $B50_{180}$ C-t-RNA, and 50_{180} C-t-RNA. The changes in optical densities were determined against a blank of uridylic acid. All samples had an initial optical density of 0.5–0.6. The solutions contained 5 mM Tris, pH 7.4, 5 mM EDTA, 6 mM magnesium chloride, and were degassed by brief vacuum treatment. The optical density was read in a cell of 1-cm light path.

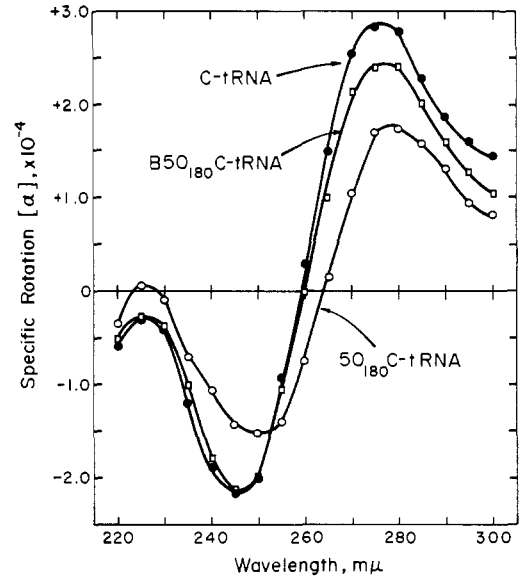

FIGURE 8: Optical rotatory dispersion of untreated and cyanoethylated *t*-RNA. The optical rotatory dispersion is plotted for the same t-RNA's described in Figure 7. The samples had about 2.0 ODU of t-RNA dissolved in a 1:4 dilution of the buffer used in Figure 7. The optical rotatory dispersions were determined in a cell of 1-cm light path at room temperature.

methoxyethanol eluate and the rest in the sodium chloride gradient although with a greatly different pattern from unreacted t-RNA. In this case it was impossible to say whether all the t-RNA molecules had been modified by cyanoethylation or whether some molecules escaped reaction and others were heavily cyanoethylated. However, in the area where unreacted RNA was eluted there were no peaks of optical density with 25_{180} C-t-RNA similar to those with unreacted t-RNA. Thus is was probable that nearly all the t-RNA molecules were uniformly reacted with one ψp cyanoethylated per t-RNA molecule.

Effect of Cyanoethylation on the Secondary Structure of t-RNA. The melting and optical rotatory dispersion curves are shown in Figures 7 and 8, with unreacted t-RNA, $B50_{180}$, C-t-RNA, and 50_{180} C-t-RNA. Although the $B50_{180}$ C-t-RNA showed some effect from incubation in the water–DMSO medium the cyanoethylated RNA showed a much larger effect. The T_m, total change in optical density, and the slope of the melting curve were all depressed. Not only was the magnitude of the change of optical rotatory dispersion reduced by cyanoethylation but also it shifted the minimum value from 245 mμ for unreacted t-RNA's to 250 mμ for all cyanoethylated RNA's. The specific rotation of control t-RNA changed from −2175 (at 254 mμ) to +2850° (at 277 mμ), a total of 5025°. The 50_{180} C-t-RNA changed 3250° between 250 and 277 mμ. This decrease in $\Delta[\alpha]$ can be accounted for by the observations of Sarin *et al.* (1966) who noted a decrease in rotation of t-RNA on loss of secondary structure with increasing temperature.

These differences between reacted and unreacted t-RNA were found only in the presence of magnesium chloride (10^{-3} or 2.5×10^{-4} M, respectively). Without Mg^{2+} present all the curves were superimposable.

The magnitude of the changes for the melting curves and optical rotatory dispersion curves are listed in Table I for t-RNA reacted for 90 and 180 min in various concentrations of water. The only control sample listed, $B50_{180}$ C-t-RNA, showed the largest effect of all the controls. It was immediately obvious that after reaction in 25, 35, and 50% water all the change in the physical properties had occurred by 90 min. Increasing the water in the reaction mixture from 25 to 50% resulted in a decrease in the secondary structure by comparable amounts. On the other hand, after incubation at the 10% water level there was a further decrease in secondary structure on continuing the incubation beyond 90 min, although even by 180 min the loss of structure was less than in 25% water after 90 min. The final extent of loss of structure at 10% water has not been determined.

Amino Acid Acceptor Activity. Figure 9 shows the acceptance of a [^{14}C]amino acid mixture by increasing amounts of t-RNA at various pH values and at various times. The acceptance of amino acids was proportional to the concentration of t-RNA throughout the range used at all pH values. The extent of incorporation reached a constant level after 1-hr incubation

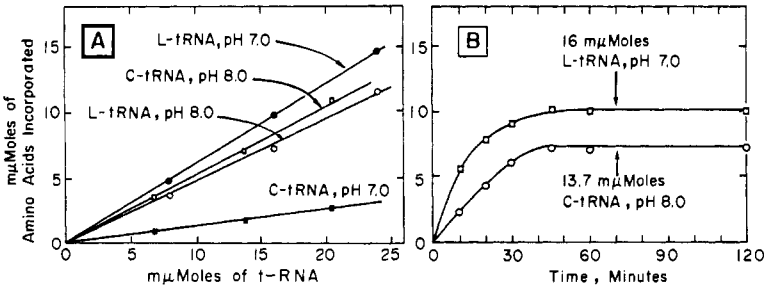

FIGURE 9: Amino acid acceptance by t-RNA. [^{14}C]Amino acid mixture (sp act. 3800 cpm/mμmole) acceptance by C-t-RNA and L-t-RNA at pH 7.0 and 8.0. (a) Acceptance after 1 hr at room temperature with increasing amounts of t-RNA, (b) kinetics of acceptance. t-RNA was determined from its optical density at 260 mμ. To 0.23 ml of solution which contained 1.13 μmoles of ATP, 0.23 μmole of amino acids, 0.3 μmole of CTP, 0.8 μmole of EDTA, 3.0 μmoles of magnesium chloride, 0.5 μmole of potassium chloride, and 80 μmoles of Tris, pH 7.0 or 8.0, were added 0.05 ml of 105,000 *g* supernatant enzyme preparation and 0.05, 0.1, or 0.15 ml of t-RNA solution. The incubations were stopped and the t-RNA was precipitated by the addition of 5.0 M NaCl and 95% ethanol. After 1 hr at −10° the precipitates were collected on 2.5-cm glass fiber filters by vacuum and washed with 80 ml of ice-cold 10% TCA. The filter pads were dried and radioactivity was determined in a liquid scintillation counter. The results were corrected for blank samples containing no t-RNA which, at pH 8.0, amounted to about 4% of the incorporation in the presence of t-RNA. The values are the average of duplicate samples which varied no more than 7% from the average.

TABLE I: Effect of Cyanoethylation of t-RNA on its Secondary Structure.[a]

			Reaction Conditions							
			10% H_2O		25% H_2O		35% H_2O		50% H_2O	
	C-t-RNA	$B50_{180}$ C-t-RNA	90 min	180 min	90 min	180 min	90 min	180 min	90 min	180 min
T_m (°C)	68.5	67.2	65.2	66.2	63.1	63.5	59.8	60.9	58.5	60.2
% total ΔOD	28.5	25.5	28.0	26.0	27.0	26.5	24.5	21.0	22.5	20.0
%ΔOD for 10°/total ΔOD	51.2	35.6	40.6	34.4	26.6	27.5	23.2	24.2	22.5	22.0
Δ[α] min − max (deg)	5025	4825	4690	4355	4145	4095	3960	3845	3505	3250

[a] The effect of cyanoethylation of t-RNA on the melting curves (Figure 6) and the magnitude of the change of [α] from the 245–250-mμ region to the 277-mμ region, Δ[α] min − max, as illustrated in Figure 7, are given. T_m, midpoint of melting; % total ΔOD, total change in optical density at 260 mμ from 25 to 100°; %ΔOD for 10°/total ΔOD, change in optical density 5° each side of T_m compared to total change in optical density.

at room temperature; about 52% of the C-t-RNA had accepted amino acids at pH 8.0. This apparent maximum acceptance represented an equilibrium value between the enzymatic charging of the RNA and a nonspecific hydrolysis and loss of amino acid from the RNA (Berg *et al.*, 1961). The greatly decreased acceptance by C-t-RNA at pH 7, 13% charged, was due to the inability of the C-t-RNA to regain its necessary terminal adenosine at this pH and the residual incorporation by C-t-RNA at this pH represented the fraction of RNA in the commercial preparation which had retained the terminal adenosine. The total amount of ATP incorporation necessary did not limit the equilibrium value for amino acid acceptance since at pH 8.0 both L- and C-t-RNA accepted about the same amount of amino acids. Consequently all cyanoethylated t-RNA's were tested for amino acid acceptor activity at pH 8.0.

The effect of cyanoethylation on amino acid acceptor activity for the [^{14}C]amino acid mixture by both C- and L-t-RNA and for individual amino acid acceptance by C-t-RNA is shown in Table II. The values are given as percentages of controls incubated for comparable times with the same content of water as the reaction solution. The only difference in reaction conditions between the control and the reaction solutions was the presence of acrylonitrile in the latter. The $B50_{180}$ t-RNA had lost about 30% of its total acceptor ability compared with the starting material. The 50% water control showed the greatest loss of acceptor ability.

For the t-RNA treated in 25–50% water it was

TABLE II: Effect of Cyanoethylation of t-RNA on its Amino Acid Acceptor Activity.[a]

	L-*t*-RNA	C-*t*-RNA						
[^{14}C]Amino acid (cpm/mμmole)	Amino acid mixture 3,800	Amino acid mixture 3,800	Tyrosine 58,200	Serine 45,500	Alanine 48,000	Valine 17,100	Lysine 32,000	Aspartic acid 48,600
Control level[b]	4.6	5.2	0.288	0.425	0.131	0.10	0.495	0.33
					Percentage of Controls			
Reaction Conditions	Expt 1 / Expt 2	Expt 1 / Expt 2 / Expt 3						
10_{60}	81	81						
10_{90}	78	73	81					
10_{180}	64 / 53	47 / 52	56					
25_{60}	43	43						
25_{90}	37	30 (32)[c]	44	51	53	35	34	41
25_{180}	39 / 30	29 (33)[c] / 28	49	58	55	41	40	46
35_{60}	38	36						
35_{90}	21	19	23					
35_{180}	24 / 25	19 / 17	24					
50_{60}	32	34						
50_{90}	19	16	22	25	24	12	5	11
50_{180}	11 / 8	10 / 8 / 12	17	18	20	9	4	7
50_{360}		4						
50_{540}		5						

[a] The effect of cyanoethylation of C-t-RNA and L-t-RNA upon its acceptance of a [^{14}C]amino acid mixture and individual [^{14}C]amino acids. The values for the reacted RNA are given as per cent of control samples incubated in DMSO with the same water content for the same length of time but without acrylonitrile. The control incubated in 50% water for 180 min was about 70% of unreacted t-RNA. The 50% water samples gave the greatest nonspecific loss of activity. The per cent water in the reaction is given by the first figure and the reaction time in minutes as a subscript. The results are the average of duplicate assays for amino acid acceptance and each assay had about 10 mμmoles of t-RNA. Corrections have been made for ^{14}C from samples containing only enzyme and no t-RNA. These corrections amounted to about 4% of the ^{14}C found in 14 mμmoles of unreacted RNA. The conditions for assaying [^{14}C]amino acid acceptance are described in the legend to Figure 9. [b] Amino acid uptake (mμmoles)/10 mμmoles of unreacted t-RNA. [c] 2-hr incubation with amino acids.

apparent that the majority of the loss of acceptor activity took place within 90 min. However, it did take the full 90 min for the initial loss to be completed. These samples did not completely lose all acceptor ability. It was also obvious that increasing the amount of water in the reaction mixture from 25 to 50% led to increasing loss of acceptor activity. This loss was not due to loss of the capacity to accept a particular amino acid since individual amino acids gave approximately the same results. Nor was the loss of acceptor activity due to loss of ability of the C-t-RNA to incorporate ATP, since the L-t-RNA, retaining three-quarters of its terminal adenosine, gave only slightly higher acceptance than C-t-RNA having about one-third of its terminal adenosine. On the other hand, t-RNA treated in 10% water continued to lose acceptor activity at a slow rate through the 180 min studied.

Discussion

It was immediately apparent that the amount of water added to the DMSO solution markedly affected the extent of cyanoethylation of the ψp in t-RNA. In 10% water (180 min) only about 0.3 ψp/t-RNA molecule was cyanoethylated whereas in 25% water at 90 min about 1.2 residues/molecule were cyanoethylated. In 35% water two ψp per molecule and in 50% water all ψp were cyanoethylated (three per t-RNA molecule). Addition of water above 25% to the system did not increase the rate of reaction since the initial fast cyanoethylation of ψp was complete in all cases at the same time, 90 min (Figures 2a and 5). Moreover, further exposure of the t-RNA in 25–50% water to cyanoethylation beyond the first 90 min did not cause a further loss of secondary structure (Table I) or biological activity (Table II). Thus, only

those ψp residues which are "exposed" react rapidly with acrylonitrile and the number "exposed" depends on the water content of the system. Although other ψp can be cyanoethylated after 90 min their rate of reaction is extremely low compared with the initial rate. In an analogous manner the amount of water controls the number of uridylic acid residues available for cyanoethylation (Figure 2b).

It is not known why certain ψp's are exposed to cyanoethylation and others are not at 25–35% water in DMSO. The favored hypothesis, currently being investigated, is that the amount of water in some way controls the secondary structure of the t-RNA, the more water added the more the t-RNA unfolds. Katz and Penman (1966) found considerable guanine–cytosine base pairing in pure DMSO and one might expect considerable base pairing in t-RNA under similar conditions. However, all attempts to date to demonstrate secondary structure of t-RNA dissolved in DMSO–water solvents have been unsuccessful. In pure DMSO there was no change in absorption of t-RNA at 280 mμ upon heating to 80°. Also the t-RNA showed no optical rotation from 270 to 330 mμ for all levels of water up to 50%. Nevertheless, if secondary structure is involved, the inability of the other ψp to be cyanoethylated at 25–35% water would indicate that they are not in rapid equilibrium with an open structure. Yoshida and Ukita (1965b) also found that the secondary structure markedly affects the amount of cyanoethylation of ψp that can take place. In an aqueous solvent at low salt concentration all the ψp can be reacted while at high salt concentration only about one ψp per t-RNA molecule is substituted, similar to our results with 25% water–DMSO.

A correlation was also apparent between the loss of both secondary structure (Table I) and biological activity (Table II) of the t-RNA and the amount of cyanoethylation of ψp; when more ψp was reacted less secondary structure and biological activity remained. The initial rapid loss of amino acid acceptor activity was not complete (60 min) until the cyanoethylation of ψp was also complete (90 min, Table II).

This loss of activity and structure could not have been due to reaction of nucleotides other than ψp. Doubling the amount of cyanoethylation of uridylic acid residues by extending the period of reaction from 90 to 180 min, (Figure 4) had no further effect on the structure and biological activity. If a particular uridylic acid residue was responsible for these losses it would have to react more rapidly than the others in the t-RNA. This particular uridylic acid residue would also have to be completely substituted in 90 min while the majority of uridylic acids would have taken 15 hr (Figure 2b) to be completely reacted. Figure 4 shows that this was not the case since the rate of cyanoethylation of uridylic acid in t-RNA was linear for at least 180 min.

That cyanoethylation of some other nucleotide, for example, guanylic or inosinic acid, was leading to loss of secondary structure and acceptor activity, was also extremely unlikely. By direct analysis the total of cyanoethylated products at 90 min differed by only 0.3 residue/t-RNA molecule from the cyanoethylation of the whole t-RNA as determined by the uptake of [^{14}C]acrylonitrile (Figure 4). Thus in 50% water after 90 min (Table II) only one out of three molecules of t-RNA could have had another nucleotide cyanoethylated whereas at least 80% of the amino acid acceptor activity of the t-RNA was affected.

The magnitude of the effects of cyanoethylation of ψp upon the properties of t-RNA was also interesting. Reaction of ψp leads to some loss of secondary structure of t-RNA but not complete loss. Increasing the reacted ψp did not decrease equally all the parameters of secondary structure studied (Table I).

Yoshida and Ukita (1965b) reported that cyanoethylation of t-RNA in 0.01 M phosphate buffer led to a lowering of T_m by 7–8°, when about 2.7 ψp were reacted/molecule. However, they found no change when the t-RNA was cyanoethylated in the same buffer which contained 0.5 M sodium chloride. They correlate this effect on T_m with the secondary structure of the t-RNA during the reaction. The t-RNA reacted in concentrated salt did not lose its secondary structure probably because the ψp's affecting secondary structure were not exposed during the reaction. In our 25% water system we are presumably reacting a ψp that is involved in secondary structure.

The biological activity of the t-RNA was sensitive to cyanoethylation. Cyanoethylation of one ψp per t-RNA molecule reduced the amino acid acceptor activity by about 70% and cyanoethylation of two by 80% and three by about 85% (Table II). When the amino acid acceptor activities of the t-RNAs were compared after cyanoethylation of 1.3 ψp/molecule in the presence of 25 and 50% water, it was found that the loss of activity in the 25% water sample was twice that of the 50%. If all the ψp were equally important for amino acid attachment, there should have been equal loss of amino acid acceptor activity. This can only mean that the ψp reacted at 25% water was more important in facilitating the attachment of amino acids. However, since further loss of acceptor activity occurred with further cyanoethylation of ψp, the other ψp not exposed at 25% water must also play a role in acceptor activity.

It was also apparent (Table II) that the loss of activity was not due to loss of any specific amino acid acceptors. Rather the loss was general to all acceptors tested although they showed somewhat different sensitivity. Further, when all the ψp in the t-RNA was reacted there still remained some residual amino acid acceptance. Thus ψp was not an absolute requirement for amino acid acceptance. It should be noted that 50_{180} C-t-RNA in which the ψp residue in the probable anticodon of tyrosine t-RNA (Madison *et al.*, 1966) would be fully blocked, still retained some tyrosine acceptor activity.

Thus cyanoethylation of ψp leads to a general

loss of activity not a specific loss. The aminoacyl-t-RNA synthetases probably need the correct secondary structure of the t-RNA molecule in order to place the amino acid efficiently on the t-RNA molecule. However, even after 180 min of cyanoethylation when all ψp in the t-RNA molecules was reacted, these enzymes could still esterify some amino acids to t-RNA. This amino acid acceptor test involves a steady-state reaction and does not indicate the absolute extent of amino acid acceptance by the t-RNA. A low steady-state value may reflect a less efficient amino acid acceptance by the t-RNA molecule. Thus all the reacted t-RNA molecules appear to be able to accept their amino acids but owing to loss of secondary structure can accept them less efficiently.

Modification of the secondary structure has no effect on incorporation of adenylyl residues from ATP in the incubation mixture used for determining acceptor activity since L- and C-t-RNA, which initially contain different amounts of terminal adenosine, accept amino acids equally well after cyanoethylation. That all t-RNA's show a similar but not complete loss of acceptor activity concurrent with loss of secondary structure after substitution of 1.3 ψp/chain in 25% water would indicate a sequence in all t-RNA's which contains a ψp in common. Modification of this particular residue greatly reduces the efficiency toward all the aminoacyl synthetases. The trinucleotide sequence (Gp)TpψpCp(Gp) found by Zamir *et al.* (1965) to be present in all t-RNA's could satisfy the observations.

Ofengand *et al.* (1966) also found when cyanoethylating t-RNA of *E. coli* in aqueous solution that "most or all amino acid accepting chains were inactivated." They also concluded that "modification of one or more ψp residues is responsible" in agreement with our results that cyanoethylation of a particular ψp was responsible for loss of activity. The principal difference between our findings and theirs is that they can "inactivate" the *E. coli* t-RNA while we find only partial loss of activity of yeast t-RNA when all ψp's are reacted. Perhaps the cyanoethylation of thiouridylic acid, which is present in t-RNA of *E. coli* but not that of yeast, accounts for the complete loss.

Next we should consider cyanoethylation of t-RNA in the 10% water solvent, where very little ψp was substituted (Figure 4). The rate of reaction was slow. The loss of secondary structure and activity was continuous and linear and was not complete over the 180 min studied (Tables I and II), unlike the other water–DMSO systems where the loss was completed by 90 min. It was, therefore, unlikely that reaction of ψp contributed to the majority of this loss. A more attractive hypothesis is that cyanoethylation of uridylic or inosinic acid residues or both also leads to loss of secondary structure and biological activity. Compared with cyanoethylation of ψp, these nucleotides reacted to a much higher proportion at 10% water than at 50% water (Figure 4). Further, cyanoethylation of these nucleotides was continuous over the period studied, in agreement with the loss of structure and activity (Tables I and II). Since there was no rapid loss of amino acid acceptor activity in the 10% water reaction where practically no ψp was cyanoethylated the rapid loss of activity noted in 25% water must be due to cyanoethylation of ψp.

It would appear that a major function of ψp in t-RNA is to maintain the secondary structure of the molecule. The studies of Pochon *et al.* (1964) demonstrated that poly ψp forms a remarkably stable secondary structure compared with polyuridylic acid. That the secondary structure of t-RNA is important to its biological activity was also demonstrated by Penniston and Doty (1963) in studies of reaction with formaldehyde. Though ψp is not involved in this reaction, the loss of amino acid acceptor activity paralleled the loss of secondary structure.

This influence of ψp is impressed on the t-RNA molecules after they have been synthesized on the DNA template (Weiss and Legault-Demare, 1965). Apparently the nascent t-RNA molecules are modified in the cytoplasm of the cell by substituting ψp into the chains to stabilize a secondary structure more efficient for amino acid acceptance. The role of ψp in other reactions of t-RNA is under investigation.

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Yeast Hexokinase. I. Preparation of the Pure Enzyme*

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ABSTRACT: A method is described for the preparation of baker's yeast hexokinase, which can be adapted for the preparation of yeast enzymes located at sites similar to that of hexokinase. It has been demonstrated by studies of the contaminating proteases that it is possible to isolate such an enzyme from yeast without apparent proteolytic degradation. This is achieved by rigorous maintenance of conditions ensuring the inhibition, including organophosphate inhibition, of the proteolytic enzymes. The procedure is then superior to previous hexokinase preparations in respect to the lack of degradation, the yield, the specific enzymic activity, the stability, and the preparation time. Two hexokinase species appear to be present under conditions of isolation, hexokinase A (in equal or larger amount) and hexokinase B. Each is a homogeneous protein by a number of criteria. Hexokinase B, as judged by criteria of homogeneity, purity, and stability, is a proteolytically undegraded enzyme having a specific activity of 800 units/mg at 25°; this is the highest reported for yeast hexokinase. Hexokinase A has a lower specific activity. Substrate specificity, chromatographic elution position, and electrophoretic mobility differences exist between these two species.

For active center and subunit studies on a kinase enzyme, baker's yeast hexokinase (ATP[1]:D-hexose 6-phosphotransferase, EC 2. 7. 1. 1) offers some advantages, but it is desirable for such studies to secure the enzyme in a homogeneous and completely undegraded form. Present methods of preparation, which involve a deliberate autolysis at 37° for its release from the yeast, create difficulties in this respect. We have examined other methods for this release, and describe here a new procedure for hexokinase purification that throughout minimizes proteolytic attack. The enzyme thus obtained is stable and homogeneous.

Yeast hexokinase was first partially purified by Meyerhof (1927) and later by N. van Heyningen;[2] crystallization was performed by Cori *et al.*[3] (Bailey and Webb, 1948). Later Kunitz and McDonald (1946), using ammonium sulfate fractionation for the first time in the hexokinase purification procedure, also isolated and crystallized the enzyme. The yields in the Kunitz and McDonald (1946) method and the Bailey and Webb (1948) modification of this procedure did not encourage large-scale studies of the enzyme. More recently, Ågren *et al.* (1963) and Darrow and Colowick (1962) have described improved methods of purification. The former workers, using commercially available enzyme, purified it by chromatography on CM-cellulose followed by chromatography on DEAE-cellulose. A sedimentation analysis of the enzyme, in 0.5% glucose at pH 7.0, yielded a single boundary with a sedimentation coefficient of 4.0 S. A determination of the molecular weight by the Archibald method gave a value of 50,000. This discrepancy with the molecular weight of 96,000 as reported by Kunitz and McDonald (1946) was thought to be due to cleavage of the molecule by glucose as had previously been

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[1] Abbreviations used in this work: ATP, adenosine 5′-triphosphate; TCA, trichloroacetic acid; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; BAEE, *N*-benzoyl-L-arginine ethyl ester; TPCK, *N*-tosyl-L-phenylethyl chloromethyl ketone; PMB, *p*-mercuribenzoate.

[2] Cited by M. Dixon, in Report to Ministry of Supply, No. 10, London, 1942.

[3] C. F. Cori, S. P. Colowick, L. Berger, and M. W. Slein (1942) cited by Bailey and Webb (1948).