The Function of Pseudouridylic Acid in Transfer Ribonucleic Acid

I. THE SPECIFIC CYANOETHYLATION OF PSEUDOURIDINE, INOSINE, AND 4-THIOURIDINE BY ACRYLONITRILE*

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SUMMARY

The ability of acrylonitrile to cyanoethylate 13 nucleosides was examined. Only pseudouridine, inosine, and 4-thiouridine were found to react rapidly, yielding N1-cyanoethyl pseudouridine, N1-cyanoethyl inosine, and S-cyanoethyl 4-thiouridine, respectively. At much slower rates, a reaction with position N1 of uridine, ribothymidine, and N1-cyanoethyl pseudouridine was observed, and at very slow rates a 2'- (or 3') cyanoethyl derivative was produced with adenosine, guanosine, cytidine, and N1-cyanoethyl inosine. The variation of reaction constant with pH showed that the rapidly reacting nucleosides must be in the anionic form for reaction to occur. The spectral, chromatographic, and electrophoretic properties of the derivatives were determined and used to assign the structures as given. The stability of the cyanoethyl derivatives to alkali and acid was examined and found to be sufficient to allow the application of standard procedures for the detailed nucleotide analysis of cyanoethylated transfer RNA.

In the biosynthesis of protein, amino acyl transfer RNA faces three critical recognition problems. They are recognition of the correct amino acyl RNA synthetase, recognition of ribosomal binding sites, and recognition of the correct codon in messenger RNA. Presumably, specific conformations of tRNA determine the selectivity of these interactions, and it seems reasonable to assume that the high content of unusual nucleotides found uniquely in tRNA may play an important role in maintaining this conformation. Most of the unusual nucleotides occur infrequently however, less than once per chain on the average, with the exception of pseudouridylic acid and ribothymidylate which are found two or more times per chain on the average and once per chain, respectively (1-5). Dihydrouridylate is also present two or more times per chain in each of the five tRNAs for which the sequence is known.

While there is little doubt that even the nucleotides which occur in only a limited number of chains have an important function, those which are to be found in every chain would appear to be of more general significance. Of these, pseudouridylic acid appears to be of greatest interest because of its location in tRNA and its chemical structure. It is the only unusual nucleotide found both in a sequence common to all chains, GprTpGpCpGp (6) and also elsewhere in the molecule. In addition, its synthesis must represent a relatively complex task for the cell since in pseudouridylate the N—C glycosidic bond has been replaced by a C—C bond (7). Nevertheless, it is synthesized in all species and is found in large amounts only in tRNA (8, 9) (with minor exceptions, 10-12). Reasoning that this unique structure and cellular localization of pseudouridylic acid must indicate an important role for this nucleotide, we have initiated studies intended to clarify its function in tRNA.

The approach chosen was to search for a method of chemically modifying pseudouridylic residues in intact tRNA in a specific and stable manner which would then allow subsequent assay for the retention of biological activity. Cyanoethylation with acrylonitrile appears to be a suitable reaction for this purpose since (a) reaction occurs with pseudouridylic at the N1 atom blocking the very group which distinguishes pseudouridylic from uridylic acid, (b) it can be carried out under conditions which preclude phosphodiester bond hydrolysis, and (c) the reagent has minimal reactivity with most other nucleotides.

In this paper, we describe the cyanoethylation of nucleosides as a model for the cyanoethylation of intact tRNA. Here we consider the kinetics and specificity of the cyanoethylation reaction for various nucleosides, the chemical characterization of the cyanoethyl nucleoside adducts, and their chemical stability. In the next paper of this series, we will describe the effect of cyanoethylation on the structure and function of tRNA.

Preliminary reports of part of this work have already ap-
peared (13-15). In addition, Chambers (16), Yoshida and Ukita (17), and Raker and Tener (18) have reported observations similar to some of the findings described here and in general agreement with them.

EXPERIMENTAL PROCEDURE

Materials

Nucleosides—Adenosine, guanosine, and cytidine were obtained from Sigma Chemical Company as Sigma grade material. Inosine, xanthosine, thymidine, and cytidine were purchased from Calbiochem as A grade compounds. Uridine (pseudouridine-free) was also obtained from Calbiochem. On chromatographic analysis in Solvent B, the uridine was shown to contain less than 0.5% of ribothymidine, but was contaminated with about 1% of pseudouridine. Pseudouridine C and B (the \( \beta \) and \( \alpha \) anomers, respectively) were purified by the method of Ofengand and Schaefer (19) from the commercial mixture of isomers also obtained from Calbiochem.

One sample of ribothymidine, prepared by the method of Fox et al. (20), was purchased from Cyclo Chemical Corporation, and another sample was isolated from E. coli transfer RNA (21) by standard methods. The RNA was digested in 0.3 \( \times \) NaOH at 37°C for 15 hours and the uridine-like nucleotides were isolated by passage through Dowex 50-H\(^+\) in 0.01 N HCl. Salt was removed by the method of Uziel and Cohn (22). After enzymatic removal of the phosphate group, ribothymidine was isolated by preparative thin layer chromatography in Solvent B which readily separates ribothymidine, uridine, and pseudouridine from each other (Table I). Both the isolated and synthesized samples of ribothymidine were homogeneous when chromatographed in Solvent C of Table I. In addition, the ultraviolet absorption spectra at pH 2 and 12 and showed a single spot of the phosphate group, ribothymidine was isolated by preparative thin layer chromatography in Solvent B which readily separates ribothymidine, uridine, and pseudouridine from each other (Table I). Both the isolated and synthesized samples of ribothymidine were homogeneous when chromatographed in Solvent C of Table I. Since both yielded \( N \)-cyanoethyl derivatives when treated with acrylonitrile (see below), they have not been further distinguished in the text.

4-Thiouridine disulfide, dihydrodurylidine, and \( N_{1,3} \)-dimethyl-4-thiouracil were obtained from Cyclo. 4-Thiouridine was prepared from the disulfide by reduction with mercuric oxide, followed by chromatographic purification in butanol-H\(_2\)O (86:14) (23). The purified product possessed the spectral properties described by Lipsett (23) (\( \lambda_{\text{max}} \) (pH 12), 317 \( \text{nm} \); \( \lambda_{\text{max}} \) (pH 2), 331 \( \text{nm} \); \( \epsilon_{127}^{127} / \epsilon_{127}^{127} \) = 0.93) and was chromatographically homogenous in Solvents A, B, and C of Table I. The commercial dihydrodurylidine was purified by preparative thin layer chromatography in t-BuOH-CH\(_3\)CO\(_2\)H-HICO\(_2\)-H\(_2\)O (92:12:15:15) (24) from ureido-reacting as well as ultraviolet-absorbing contaminants. After elution, the product appeared homogenous when chromatographed in sec-BuOH saturated with H\(_2\)O, t-BuOH-CH\(_3\)CO\(_2\)H-H\(_2\)O (9:8:1) and Solvent D.

\( N_{1,3} \)-dimethyl-4-thiouracil, prepared by the method of Elion and Hitchings (25), was chromatographically homogenous in solvent systems A, B, and C (Table I). In addition, the ultraviolet spectrum was invariant over the pH range 2 to 12, showing the absence of any ionizable protons, and was very similar to a slightly displaced acid spectrum of 4-thiouridine (\( \lambda_{\text{max}} \) 328 \( \text{nm} \)).

2-Thiouracil was the gift of Dr. John Carbone. It was chromatographically homogenous in Solvents B and C.

Cyanoethyl Nucleosides—In general, cyanoethyl derivatives were synthesized by the procedure previously described (13). A reaction mixture was prepared containing nucleotide at a concentration of 0.04 \( \mu \) or less, 1 \( \mu \) acrylonitrile, and 0.05 \( \mu \) sodium carbonate buffer, pH 9.6 to 10. After incubation for an appropriate time at 37°C in well sealed tubes, the reaction was terminated by the adjustment of the pH to 6 to 7 with HCl. In some cases, carbonate buffer was omitted, and the pH held constant by periodic additions of acid or alkali. Purification of the desired compound from the reaction mixture was achieved by thin layer chromatography in the solvent which gave optimal separation of the unreacted nucleoside from its cyanoethyl adduct (see Table I). In some cases, multiple development with the same or different solvents was used. CE-nucleosides were recovered by elution with water. When subsequent spectral analysis showed the presence of a contaminant ionizable at alkaline pH, purification by chromatography in Solvents A or B was used, since in this alkaline medium, the unionizable CE-nucleosides separated well from ionizing contaminants. In addition, the purity of all CE-nucleosides was assessed by chromatography in Solvents B, C, and E and was greater than 95%.

Other Chemicals—Acrylonitrile was obtained from Matheson, Coleman, and Bell. After redistillation it was stored in the cold. Except as otherwise indicated, all other chemicals used were of reagent quality.

Methods

Chromatography—Ascending thin layer chromatography on layers of cellulose (0.5 to 1 mm) was performed as described previously (19). In most cases, volatile solvents were used to permit multiple development with the same or different solvents in order to obtain improved resolution of compounds with similar \( R_F \) values. The \( R_F \) values in several solvents for all of the CE-nucleosides and related compounds described in this paper are summarized in Table I.

Kinetic Measurements—The rate of reaction of nucleoside with acrylonitrile was determined by one of two methods.

1. Spectral technique. This method was used for rapidly reacting compounds. Buffer (20 ml), usually 0.05 \( \mu \) carbonate or pyrophosphate plus NaCl or MgOAc as indicated, was prepared at the desired pH and the tare weight was determined. Acrylonitrile was then added with stirring, and after solution was achieved the flask was reweighed to determine the true amount of acrylonitrile added. In all cases, kinetic measurements were made with solutions approximately 1 \( \mu \) in acrylonitrile. (There is less than 0.5% volume contraction on mixing these quantities of acrylonitrile and water.) After readjustment of the pH at 30°C, the solution was placed in both reference and sample cells and equilibrated.

The reaction was started by the addition of nucleoside in 1% or less of the total volume. pH measurements were taken before and during the course of the kinetic run and did not vary by more than 0.04 unit. The average value was used. Recordings were made either as complete spectra on a Cary model 14 spectrophotometer as a function of time (see Figs. 1 to 4) or automatically at a single wavelength in a Gilford model 2000/Beckman DU monochromator system. Both methods gave equivalent results (see Figs. 5 to 8). A pseudo first order plot of the absorbance change, \( D_t - D_o \) was used according to Equation 15 or 16 (see the “Appendix”) to determine \( k_{\text{app}} \). Alternatively, the method of Guggenheim (26) was used when the reaction rate was too slow to allow completion of the reaction to be conveniently reached, or when a second slower reaction followed the
Both methods were used for calculation in some cases and yielded
Pseudouridine C: 0.03 0.40 0.09 0.59 0.40
reagent according to Lipsett (23). Dihydrouridine was detected
Pseudouridine B: 0.05 0.50 0.11 0.69 0.44
CE-pseudouridine B: 0.05 0.36 0.11 0.51 0.47

The rapid initial one, as is the case in the reaction with pseudouridine
(Figs. 1 and 2). In this method, \( D_t - D_{t+\Delta t} \) is substituted for
\( D_1 - D_0 \), where \( \Delta t \) is a fixed time interval for the experiment.
Both methods were used for calculation in some cases and yielded
the same \( k_{app} \) within 5%.

2. Thin layer chromatography method. This method was
used in instances in which no spectral shift was anticipated or in
which reaction was expected to be very slow or nonexistent. A
mixture of buffer, acrylonitrile, and nucleoside was prepared as
above at a given pH and incubated at 30°C. Acrylonitrile
was omitted in the control tube. All spots were removed as a
function of time, neutralized with HCl, and frozen. After chroma-
tography in a suitable solvent (see Table I), the spots were eluted
above at a given pH and incubated at 30°C. Acrylonitrile was
omitted in the control tube. All spots were removed as a
function of time, neutralized with HCl, and frozen. After chroma-
tography in a suitable solvent (see Table I), the spots were eluted
and quantitated by absorbance measurements with suitable cor-
rections for blanks and elution efficiencies. The amount of
nucleoside remaining was determined directly and also by differ-
ence by measuring the amount of CE-nucleoside produced. \( k_{app} \)
was determined from the usual first order plot of remaining nu-
cleoside versus time according to Equation 12 of the “Appendix”
(see Fig. 9).

In the one case where the two kinetic methods were compared
(see Table III, line 3, below), the same constant was obtained in
both cases.

\( pK \) Constants—\( pK \) determinations were made by the spectral
method as previously described (19). For pseudouridines C
and B, the values are 8.97 and 9.19, respectively (19). For
inosine, the value found, 8.83, was in agreement with the litera-
ture value of 8.8 (27). For 4-thiouridine, a value of 8.00 was
determined. Lipsett has reported 8.25 (23). For ribothymidine,

Other Methods—Thiol and thioketone functional groups were
detected by the iodine-azide method of Feigl (20) used as a spray
reagent according to Lipsett (23). Dihydrouridine was detected
by the method of Fink et al. (30). pH was measured to 0.01 pH
unit with a Radiometer pHM20S meter with electrodes stand-
ardized against pH 7.00 and 10.00 Beckman standard buffers at
the temperature of measurement. All spectral kinetic measure-
ments were made in stoppered cells sealed with paraffin and
thermostatted at 30°C.

**RESULTS**

Kinetics and Specificity of Cyanoethylation

Pseudouridine, Inosine, and 4-Thiouridine—In studies pre-
viously reported (13), it was shown that reaction with acryloni-
trile yielded a stable nucleoside adduct which retained the spec-
trum characteristic of the nucleoside at acid pH even though the
solution was made alkaline. This property suggested that it
would be possible to follow the cyanoethylation of nucleosides
directly by spectral techniques since the reaction is usually

carried out at a pH above the \( pK \) of the nucleoside, and these nucleo-
sides all have sufficiently different acid and alkaline spectral
curves. This prediction was borne out in the experiments shown
in Figs. 1 to 4, which show the effect of cyanoethylation on the

**TABLE I**

Chromatographic behavior of nucleosides and cyanomethyl nucleosides

| Compound                          | Rf in solvent |
|----------------------------------|--------------|
|                                  | A     | B     | C     | D     | E     |
| Pseudouridine C                  | 0.03  | 0.40  | 0.09  | 0.59  | 0.40  |
| CE-pseudouridine C               | 0.06  | 0.50  | 0.13  | 0.09  | 0.49  |
| Pseudouridine B                  | 0.29  | 0.61  | 0.21  | 0.84  | 0.81  |
| CE-pseudouridine B               | 0.64  | 0.38  | 0.09  | 0.55  | 0.34  |
| Pseudouridine C                  | 0.05  | 0.50  | 0.11  | 0.69  | 0.44  |
| CE-pseudouridine B               | 0.30  | 0.62  | 0.20  | 0.86  | 0.82  |
| Inosine                          | 0.05  | 0.36  | 0.11  | 0.51  | 0.47  |
| CE-inosine                       | 0.23  | 0.46  | 0.17  | 0.63  | 0.68  |
| CE,inosine                       | 0.36  | 0.56  | 0.24  | 0.73  | 0.79  |
| 4-Thiouridine                    | 0.14  | 0.69  | 0.32  | 0.27  | 0.49  |
| CE-4-thiouridine                 | 0.57  | 0.68  | 0.41  | 0.86  | 0.85  |
| Uridine                          | 0.11  | 0.37  | 0.20  | 0.58  | 0.51  |
| CE-uridine                       | 0.48  | 0.59  | 0.33  | 0.81  | 0.87  |
| Ribothymidine                    | 0.27  | 0.61  | 0.30  | 0.77  | 0.63  |
| CE-ribothymidine                 | 0.67  | 0.70  | 0.55  | 0.92  | 0.86  |
| Adenosine                        | 0.32  | 0.59  | 0.26  | 0.65  | 0.61  |
| CE-adenosine                     | 0.46  | 0.70  | 0.43  | 0.78  | 0.71  |
| Guanosine                        | 0.04  | 0.35  | 0.16  | 0.48  | 0.32  |
| CE-guanosine                     | 0.09  | 0.48  | 0.28  | 0.68  | 0.46  |
| Cytidine                         | 0.16  | 0.37  | 0.13  | 0.52  | 0.51  |
| CE-cytidine                      | 0.33  | 0.48  | 0.25  | 0.98  | 0.71  |
| Thymidine                        | 0.59  | 0.69  | 0.84  | 0.71  |
| CE-thymidine                     | 0.71  | 0.71  | 0.93  | 0.71  |
| Dihydrouridine                   | 0.71  | 0.93  | 0.52  | 0.71  |
| Xanthosine                       | 0.39  | 0.71  | 0.93  | 0.71  |
| 2-Thiouridine                    | 0.51  | 0.25  | 0.39  |
| 1,3-Dimethyl-4-thiouracil        | 0.82  | 0.92  | 0.80  | 0.71  |

*FIG. 1.* Spectral changes accompanying the cyanoethylation of
pseudouridine C with acrylonitrile as a function of time. The
kinetic measurements were performed by the spectral technique
as described in “Methods.” Sodium carbonate buffer (0.05 M)
was used.
spectra of pseudouridine C and B, inosine, and 4-thiouridine as a function of time. There are several features common to all four of these reactions. First, each set of spectral curves shows clear isosbestic points, a good indication of a single reactant-product system without spectral complications. The shift in the pseudouridine spectral curves at later times is due to the secondary slow conversion of N1-CE-pseudouridine to N1,N5-CE-pseudouridine (13) (note especially the dashed curves). Second, the rate of reaction with time is accurately pseudo first order in each case. Third, in the case of inosine at least, the final spectrum obtained is the same as that of the chromatographically isolated reaction product. The reaction does not come to a stop because an equilibrium is reached (except in the case of 4-thiouridine at high pH) since all four of the adducts can be isolated and show no signs of decomposition when reincubated under the same conditions in the absence of acrylonitrile. (CE-4-thiouridine shows some decomposition, see below.) Also, the reaction of both pseudouridine B and C is not due to isomerization of one to the other (7), since incubation of each isomer separately at pH 10 for 24 hours in the absence of acrylonitrile resulted in no evidence for a rate of isomerization of either isomer which was more than 1% of the cyanoethylation rate seen here. In this experiment, conversion of isomers was measured spectrally at pH 12 by taking advantage of the distinct alkaline spectra of the two forms (7, 19).

In order to examine more carefully the hypothesis proposed previously (13) that ionization must precede reaction of a nucleoside with acrylonitrile, the rate of reaction was measured as a function of pH. The predicted rate expression for the general case is derived in the “Appendix” and is given in Equation 12, and in Equation 15 in a more useful form. From this derivation it is clear that the apparent form of the reaction will be pseudo first order (as is observed) and $k_{app}$ will vary with pH in a way which depends on the values chosen for $k_1$, $k_5$, $k_a$, and $k_r$. $k_5$ was known for 4-thiouridine and was negligible for the other nucleosides, while $k_1$ was negligible for all of the nucleosides (see below). However, no a priori relative estimate of $k_1$ and $k_2$ was available. In order to evaluate the experimental data provided in Fig. 5 to 8, two cases were considered. In the first case, $k_1 = 0$. That is, reaction only occurs via the ionized nucleoside. In the other case, 20 to 30% of the reaction was assumed to proceed via the unionized nucleoside, i.e. $k_1 = 0.2$ to 0.3 $k_r$. The theoretical curves for each case were then obtained in the following way and compared with the experimental values.

For each $k_{app}$, a $k_a$ could be calculated by means of Equations 13 and 14, since $pK$ and $k_a$ were known, and $k_r$ was defined in terms of $k_r$. The average value of $k_r$ was computed in this way, using all of the data, and then used to construct the theoretical curves shown in Figs. 5 to 8 by going back through Equations 13 and 14 again to obtain $k_{app}$ (calculated).

A comparison of the curves with the data indicates that, in every case, the best fit is obtained for $k_1 = 0$, although there is sufficient experimental scatter to prevent the detection of about 10% reaction via the unionized nucleoside. It should also be noted that different buffers, changes in ionic strength, or the presence of Mg ions have no effect on the kinetics observed here. This is true for all of the nucleosides, although it has been most
used, as exemplified in Fig. 9, which shows the rate of reaction of ribothymidine at a single pH. In this case the reaction was very slow with a rate constant about like that for uridine (Table III).

In similar experiments, the reaction of acrylonitrile with xanthosine, dihydrouridine, and 2-thiouridine was studied by the thin layer chromatography method, and reaction with orotidine by the spectral method. The results of these experiments plus data on the reaction of adenosine, guanosine, and cytidine are summarized in Table III.

Characterization of Cyanoethyl Nucleosides

Spectral Properties—In view of the mechanism of the cyanoethylation reaction presented above, it was expected that a stable cyanoethyl group would become substituted for the ionizable proton of the heterocyclic ring, resulting in a product the spectrum of which would no longer be affected by pH changes. This prediction was borne out with every nucleoside studied except adenosine, guanosine, and cytidine.

Other Nucleosides—An examination of a number of other minor nucleosides known or likely to be found in tRNA was carried out to see if still other highly reactive nucleosides existed. For these experiments, the thin layer chromatography method was extensively studied with inosine (Fig. 8 and Table II). In addition, Table II shows that inosinic 5'-phosphate with or without Mg reacts at the same rate as inosine.

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Chromatographically purified CE-inosine has a spectrum very similar to that of the acid form of inosine (see Fig. 4, curve) and is unaffected by pH changes from 2 to 11. The same thing is true of CE₂-inosine formed slowly as a secondary product in the reaction. (Evidence presented below indicates that the second cyanoethyl group adds to the 2'(3')-hydroxyl of ribose.) Similarly, the spectra of CE-uridine (13) and CE-ribothymidine (Table IV) resemble that of the unionized nucleosides and do not vary over the pH range 2 to 12.

The spectrum of CE-4-thiouridine (Fig. 3, D curve) is also invariant over the pH range 2 to 10,6, indicating that here also replacement of the ionizable proton has occurred. (The pK for 4-thiouridine is 8.0.) It was not possible to make spectral measurements at higher pH because of the rapid rate of alkaline hydrolysis. In all other cases studied, the spectrum at pH 12 and 30° was stable for at least 20 min. Unlike the previous examples, however, the spectrum of CE-4-thiouridine does not resemble that of the unionized form of 4-thiouridine. This is because cyanoethylation occurs not on N₃ but on the sulfur atom instead (see below).

The spectral variation with pH of the pseudouridine adducts was also as expected, bearing in mind that cyanoethylation at the N₁ atom would produce a CE-nucleoside with spectral properties like that of uridine which is also an N₁-substituted uracil. That is, there should be a decrease in extinction coefficient with pH due to ionization at N₁, but virtually no shift in the wavelength of maximum absorption, in marked contrast to the 24 mp bathochromic shift seen at pH 12 with pseudouridine itself (Fig. 10). Moreover, the pK calculated for the remaining ionization, 9.2 to 9.3, was very close to that for uridine, 9.25.

The data from this figure can also be used to calculate the extinction coefficient (ε) for both CE-pseudouridine C and CE-pseudouridine B as follows.

1. ε at any wavelength and pH for both isomers of pseudouridine can be obtained by combining the spectral data of Ofengand and Schaefer (19) with the ε values given by Shapiro and Chambers (31) for pseudouridine C at pH 7 and by Chambers (32) for pseudouridine C and pseudouridine B at pH 12.

2. From the reaction with acrylonitrile shown in Figs. 1 and 2, an isosbestic point can be located at the given pH of the reaction where $e_{CE-p}$ is equal to $e_{p}$ which can be determined as in (1).

3. The data of the above figure (Fig. 10) can then be used to convert this $e_{p}$ into a more useful value, $e_{max}$. When this was done, a value of 9800 was obtained for pseudouridine C, with a corresponding $e_{max}$ of 9900 for pseudouridine B. For comparison, the value for uridine is 9900 (28). These values are in good agreement with that reported for the N₁-cyanoethyl mixed isomers of pseudouridine (9100) by Yoshida and Ukita (17).

As indicated above and previously (13), monocyanoethyl pseudouridine can react further to give a presumed diacyanoethyl product which is chromatographically and electrophoretically distinct (see below). Presumably, reaction occurs at the availa-

![Fig. 8. The variation of $k_{app}$ with pH for inosine.](http://www.jbc.org/)
TABLE III

Reaction rate constants of nucleosides with acrylonitrile at 30°C

\( k_a \) is defined in the "Appendix." \( k_{\text{app}} \) was calculated from the relation \( k_{\text{app}} = k_a/(1 + 10^{10-\text{pH}}) \) as derived in the "Appendix." 

\( t_1 \) was calculated from \( k_{\text{app}} \) for 1 M acrylonitrile.

| Reaction type and method | Nucleoside | \( k_a \) (min\(^{-1} \times 10^2\)) | \( k_{\text{app}} \) (pH 9.3) | \( t_1 \) (pH 9.3) |
|-------------------------|-----------|---------------------------------|-----------------|-----------------|
| Fast                    |           |                                 |                 |                 |
| Spectral                | Pseudourine C | 16.3                           | 11.1            | 63              |
| Spectral                | Pseudouridine B | 17.3                           | 9.8             | 71              |
| TLC\(^a\)               | Pseudouridine C + B mixture | 18.2\(^b\)                   |                 |                 |
| Spectral                | 4-Thiouridine | 4.85                           | 4.6             | 152             |
| Spectral                | Inosine    | 16.8                           | 12.5            | 56              |
| Slow                    | TLC        | Uridine                        | 0.73\(^b\)     | 0.39            | 1,800           |
| TLC                    | Ribothymidine | 0.47                           | 0.15            | 4,800           |
| TLC                    | Thymidine  | 0.48\(^b\)                     | 0.12            | 6,000           |
| Spectral                | Orotidine  | 0.49                           | 0.41            | 1,700           |
| Very slow               | TLC        | Adenosine                      | 0.12 (pH 9.7)   | 6,000 (pH 9.7)  |
| TLC                    | Guanosine  | 0.04 to 0.11 (pH 9.7)           | 0 to 9,000 (pH 9.7) |
| TLC                    | Cytidine   | 0.07 to 0.09 (pH 9.7)           | 7 to 9,000 (pH 9.7) |
| Undetectable           | TLC        | 2-Thiouridine                  | <0.01 (pH 10)   | >50,000 (pH 10) |
| TLC                    | Xanthosine | <0.06 (pH 9.3)                 | >12,000 (pH 9.3) |
| TLC                    | Dihydrouridine | <0.01 (pH 10)               | >70,000 (pH 10) |

\(^a\) TLC, thin layer chromatography.
\(^b\) Data taken from a previous report (13).

TABLE IV

Spectral properties of cyanoethyl ribothymidine compared with ribothymidine as a function of pH

| Nucleoside | pH | \( \lambda_{\text{max}} \) | \( \lambda_{\text{min}} \) | \( \Delta \lambda_{\text{max}} \) |
|-----------|----|----------------|----------------|----------------|
| Cyanoethyl ribothymidine | 2.0 to 6.0 | 267 | 237 | 3.79 |
|            | 12.0 | 267 | 237 | 3.44 |
| Ribothymidine\(^a\) | 2.0 to 6.0 | 267 | 235 | 3.50 |
|            | 12.0 | 267 | 248 | 1.11 |
| Ribothymidine\(^b\) | 3.1 | 267 | 236 | 3.85 |
|            | 12.0 | 267 | 245 | 1.20 |

\(^a\) Isolated from E. coli tRNA as described in "Experimental Procedure."
\(^b\) Data from Fox et al. (20).

The findings obtained above can be contrasted with the following examples which indicate that cyanoethylation can occur without modifying spectral properties. This was the case for the reaction with adenosine, cytidine, and guanosine. Table V shows that the spectral behavior of these derivatives was unaffected by cyanoethylation and suggests that addition had occurred elsewhere in the molecule.

Chromatographic Behavior—The chromatographic properties summarized in Table I also provide some insight into the structure of the derivatives synthesized. First, it is clear that Solvent C and to a lesser extent D is able to distinguish mono- from dicyanoethyl derivatives by virtue of the increased affinity of the nucleoside for the mobile organic phase due to the addition of cyanoethyl groups. Thus, both CE\(_2\)-pseudouridine and CE\(_2\)-inosine move further than their corresponding mono-cyanoethyl derivatives even though pseudouridine has both groups on the ring and inosine has one on the ribose moiety. Second, the lack of ionizability predicted from the failure to show spectral shifts with pH can be seen directly in every applicable instance by comparison of the \( R_F \) values for the CE-nucleoside in Solvent C (isobutanol-water) versus Solvent A (butanol-NH\(_4\)-water) with the same \( R_F \) values for the parent nucleoside. As an example, consider 4-thiouridine. While the \( R_F \) goes down from 0.32 in Solvent C to 0.14 in the NH\(_4\)-containing Solvent A owing to ionization, the \( R_F \) of the CE-nucleoside actually increases in going from Solvent C (0.41) to Solvent A (0.57).

Electrophoretic Mobility—Paper electrophoresis in the presence and absence of borate was performed in order to detect cyanoethylation of the 2'(3')-hydroxyl group since only nucleosides possessing free vicinal hydroxyl groups can form a complex with borate and so acquire increased negative charge (Table VI, Experiment 1). Clearly this has occurred with cytidine, adenosine, and guanosine (Experiment 2) and with CE\(_2\)-inosine (Experiment 3, line 8). Equally clearly, no 2'(3')-O-cyanoethyl derivatives were produced from ribothymidine, 4-thiouridine, or uridine.
dine, nor in the formation of CE-inosine. Although the increased mobility due to borate is less marked in the pseudouridine series because of the greater extent of ionization at this pH, Experiments 5 and 6 show clearly that in neither the mono- nor dicyano-

Table V  
Spectral comparison of cyanoethyl adenosine, cytidine, and guanosine with their parent nucleosides

| Nucleoside | pH  | Unreacted nucleoside | Cyanoethyl nucleoside |
|------------|-----|----------------------|-----------------------|
|            | Unreacted | Cyanoethyl | Unreacted | Cyanoethyl |
| Cytidine   | 2.0  | 2.06                 | 2.02                  | 0.45       | 0.47       |
|            | 7.0  | 0.94                 | 0.92                  | 0.86       | 0.85       |
| Adenosine  | 2.0  | 0.22                 | 0.24                  | 0.86       | 0.87       |
|            | 7.0  | 0.15                 | 0.18                  | 0.79       | 0.81       |
| Guanosine  | 1.0  | 0.69                 | 0.72                  | 0.97       | 0.99       |
|            | 7.0  | 0.68                 | 0.70                  | 1.18       | 1.14       |
|            | 11.0 | 0.50                 | 0.65                  | 0.91       | 0.97       |

Additional evidence for the lack of ionizability was obtained by comparing the mobilities of the CE-nucleosides with their parent compounds in the absence of borate since these runs were performed at a pH at which the parent nucleosides were partially ionized. The results were particularly clear with CE-inosine, CE-4-thiouridine, CE-uridine, and CE-pseudouridine and show that cyanoethylation markedly reduced the electrophoretic mobility. Since ribothymidine itself is only slightly ionized at this pH, the effect is less striking. In a converse way, since CE-pseudouridine itself is approximately half-ionized at this pH, only a small effect is to be expected from the slightly lower pK of pseudouridine compared to CE-pseudouridine.

Structure of Cyanoethyl 4-Thiouridine—Although the spectrum of this derivative was stable to pH, the marked difference between it and the unionized spectrum of 4-thiouridine suggested that in this case the cyanoethyl group had been added to an atom other than the N7 originally carrying the ionizable proton. All of the possible structures are shown in Fig. 11, bearing in mind that the ionizable proton must be replaced by a stable cyanoethyl group. Originally, Structure I was expected by analogy with the other nucleoside derivatives, but the observed spectrum did not support this view. As a control the spectrum of N1,3-dimethyl-4-thiouracil was examined and proved to be very similar to the acid form of 4-thiouridine with a λmax at 328 nm instead of 335 nm but stable to pH changes from 2 to 12.

Since the derivative spectrum was similar to that of 4-thiouridine disulfide, it was important to rule out Structure IV occurring by some unusual oxidative reaction catalyzed by acrylonitrile. This was done in two ways. First, the kinetics of formation observed in Fig. 3 were first order in 4-thiouridine while an oxidative reaction should be second order. Second, exposure of the isolated derivative to 0.1 M 2-mercaptoethanol for 45 min at pH 7.7 had no effect on the spectrum. 4-Thiouridine disulfide is reduced almost immediately by this procedure (23).

It was possible to rule out both remaining structures, I and III, on the basis of the iodine-azide reaction of Feigl, since this reaction is catalyzed both by thiols and thioethers, but not by

Fig. 10. Spectrum of CE-pseudouridine isomers as a function of pH. A, CE-pseudouridine C; B, CE-pseudouridine B. Spectral measurements were made as described in “Methods.” pH 8.65 and 8.70, pyrophosphate buffer; pH 9.59 and 10.19, carbonate buffer. pK for CE-pseudouridine C, 9.2 ± 0.1; pK for CE-pseudouridine B, 9.28 ± 0.01.

It was possible to rule out both remaining structures, I and III, on the basis of the iodine-azide reaction of Feigl, since this reaction...
thioethers. The experiment is given in Fig. 12. 4-Thiouridine was used as an -SH control since it can enolize to this form, and the fully blocked dimethyl-4-thiouracil was used as a thioke-tone control to show that thikethones incapable of enolization can also react. β-(2-Thienyl)-alanine was included as an additional control. It is quite clear from an examination of the left of Fig. 12 (Before Alkali) that CE-4-thiouridine contained neither an available thiol nor thioketone functional group. However, in order to be sure that it contained sulfur at all, the experiment shown on the right of Fig. 12 (After Alkali) was performed. It was known that the 4-thiouridine derivative was unusually alkali-labile (see below) and on hydrolysis regenerated a spectrum identical with that of 4-thiouridine itself. While this alone is good evidence that 4-thiouridine had not been destroyed by cyanoethylation, the experiment shown in Fig. 12 offers confirmatory evidence for the regeneration by alkali of the sulfur-containing reactive group. It is concluded from these experiments that Structure II is the correct one for CE-4-thiouridine.

Additional confirmation comes from a study of the infrared spectrum of CE-4-thiouridine in D2O which resembles that of 4-thiouridine disulfide and the anion of 4-thiouridine (thiol types), but differs from that of unionized 4-thiouridine and N1, N3-dimethyl-4-thiouracil (thiokeitone types).

**Chemical Stability**

*CE-4-thiouridine—The alkaline lability of CE-4-thiouridine can be readily shown spectrophotometrically since it results in the generation of exactly the same set of curves shown in Fig. 3, except in the reverse time sequence. Using the spectral change at 325 μm as a measure of the loss of CE-4-thiouridine, the rate of hydrolysis was determined at 30° and several hydroxide concentrations. A general hydrolysis equation for CE-4-thiouridine can be derived from the sum of Equations 2 and 3 of the “Append-

Table VI

| Nucleoside          | Relative distance traveleda |
|---------------------|-----------------------------|
|                     | No borate | Plus borate | Δ Borate |
| Ribothymidine       | 2.4       | 9.5         | 7.1      |
| Thymidine           | 2.0       | 2.4         | 0.4      |
| Cytidine            | 0.5       | 6.4         | 6.0      |
| CE-cytidine         | 0.5       | 0.5         | 0.0      |
| Adenosine           | 1.9       | 7.3         | 5.4      |
| CE-adenosine        | 1.2       | 1.4         | 0.2      |
| Guanosine           | 5.8       | 8.6         | 2.8      |
| CE-guanosine        | 5.0       | 3.4         | -1.6     |
| Inosine             | 8.7       | 11.4        | 2.7      |
| CE-inosine          | 0.5       | 7.6         | 7.1      |
| CE2-inosine         | 0.4       | 0.5         | 0.1      |
| Ribothymidine       | 1.1       | 8.0         | 6.9      |
| CE-ribothymidine    | 0.6       | 7.4         | 6.8      |
| 4-Thiouridine       | 11.6      | 13.7        | 2.1      |
| CE-4-thiouridine    | 0.7       | 7.3         | 6.6      |
| Uridine             | 6.6       | 8.9         | 2.3      |
| CE-uridine          | 0.4       | 6.9         | 6.5      |
| Pseudouridine C     | 7.4       | 9.8         | 2.4      |
| CE-Pseudouridine C  | 6.1       | 8.1         | 2.0      |
| CE2-pseudouridine C | 0.4       | 8.0         | 7.6      |
| CE-pseudouridine C  | 6.7       | 10.8        | 4.1      |
| Pseudouridine C     | 7.0       | 11.8        | 4.8      |

* Relative to an endosmosis marker of N1, N3-dimethyl uracil. All relative movement was to the positive electrode.
The structures of the derivatives produced have been shown by acrylonitrile for pseudouridine, inosine, and 4-thiouridine have also been reported by Yoshioka and Uchida (17) in a brief communication. They also observed that $k_{app}$ increased with pH in an approximately sigmoid manner, but they did not attempt to compare their results with a theoretical prediction. The approximately three-fold higher $k_{app}$ values reported by them are probably due to the use of a higher temperature and a higher but unspecified acrylonitrile concentration.

The kinetic specificity shown by acrylonitrile for pseudouridine, inosine, and 4-thiouridine has proven useful in studies on the function of these minor nucleotides in tRNA. In addition, the fact that $k_{app}$ depends on both solution pH and nucleotide pK has been of value in studies using cyanoethylation as a chemical probe of the involvement of these minor nucleotides in secondary structure. For example, one would predict that the well known shift of pK to higher values for nucleotide residues involved in the secondary structure of RNA would markedly decrease the observed reaction rate from that expected on the basis of nucleosides or denatured RNA, and conversely, that residues in a denatured loop should react at expected rates. Such an effect has been observed (36) and will be the subject of a separate communication.

The structures of the derivatives produced have been shown by a combination of spectral, chromatographic, and electrophoretic measurements. Chromatography and electrophoresis showed
(a) the number of cyanoethyl groups added, (b) the physical loss of ionizability following cyanoethylation which had been inferred from the spectral measurements, and (c) those derivatives which had been cyanoethylated on ribose. These analyses also ruled out the possible hydrolysis of the nitrile group to a carboxyl function during the course of the reaction or isolation since such a conversion would be expected to add a negative charge to the molecule at these pH values and none was observed.

Analysis of the spectra was then sufficient to localize the cyanoethyl group to the appropriate atom of the heterocyclic ring. Assignment of the cyanoethyl group to the N1 or N4 atom of pseudouridine, uridine, and ribothymidine rather than to the O6 or O4 atom, was based on a comparison of the ultraviolet spectra at different pH values with those of the model compounds studied by Shugar and Fox (28), using uracil as a model for pseudouridine. Thus N1-methyl uracil, but not 2-ethoxy, 4-ethoxy, or N4-methyl uracil, showed spectral shifts and $\lambda_{\max}$ changes with pH like those of N1,4-CE-pseudouridine, and CE4-pseudouridine, CE4-uridine, and CE4-ribothymidine have spectra similar to N1,s-dimethyl uracil but not to N1-methyl-4-ethoxy uracil. More rigorous evidence for the absence of enolic cyanoethyl derivatives should be obtainable by infrared spectral analysis of the ultraviolet spectra was not adequate in this case.

Measurement of the infrared spectrum in D2O showed a very strong band at 1697 cm$^{-1}$, very similar in frequency, shape, and intensity to that previously found for N1-methyl inosine, but no strong band at 1687 cm$^{-1}$, corresponding to the ketonic structures differ markedly from the enol type, but this has not been done so far. However, this method was used to show the absence of an enolic structure in CE-inosine since analysis of the ultraviolet spectra was not adequate in this case.

The chemical stability of the pyrimidine derivatives are also consistent with the known behavior of N-substituted nucleoside compounds. Thus, N1,CE-pseudouridine was quite stable to alkaline decomposition, as is also the case for N1-s-dimethyl uracil (33). Moreover, the alkaline stability of CE-ribothymidine correlates well with the stability observed for N1,s-dimethyl thymine (33). The different nature of the alkaline decomposition products of CE4-pseudouridine C and CE4-pseudouridine B are no doubt related to the different orientation of the uracil rings with respect to the ribose in the two cases, but no attempt has been made as yet to rationalize the different hydrolysis products on this basis.

It is highly unlikely that under the conditions used here anionic polymerization of acrylonitrile on a cyanoethyl nucleoside could occur since the pK of the $\alpha$-hydrogen of acrylonitrile is greater even than that of water (39) and the maximum pH used in these studies was 10. Moreover, extra aliphatic groups should be detectable by chromatography in Solvents C or D. However, even in the case of reaction with pseudouridine to which at least one cyanoethyl group must be added to each N atom in view of the spectral shifts produced, chromatography in Solvent D of a reaction mixture at different times of reaction provided no evidence for compounds other than the expected ones. Had polymerization on the cyanoethyl group occurred, additional nucleoside products would have been expected, but none were detected. Thus it appears that the added group in all the cases described here is a monocyanocarbonyl group.

The number of cyanoethyl groups added in the various CE-nucleosides described here is emphasized because of other studies in which the specific radioactivity associated with the cyanoethyl group was used to estimate the number of particular CE-nucleosides in cyanoethylated transfer RNA.2

In conclusion, the cyanoethylation reaction described in this paper appears to be a useful one for a variety of functional and chemical studies on "minor" nucleotides in transfer RNA.

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APPENDIX

A. Rate Expressions for Reactions

\[
\begin{align*}
\text{NH} & \xrightleftharpoons[K]{H}[\text{H+}] \text{N}\text{H}^+ + \text{H}^- \\
\text{NH} + \text{A} & \xrightleftharpoons[k_1]{k_2} \text{NAH} \\
\text{N}\text{H}^+ + \text{H}_2\text{O} & \xrightleftharpoons[k_3]{k_4} \text{NAH} + \text{OH}^- \\
\end{align*}
\]

where $\text{NH}$ and $\text{N}\text{H}^+$ are the un-ionized and ionized nucleosides, respectively, $\text{A}$ is acrylonitrile, and $\text{NAH}$ is the cyanoethyl nucleoside.

It is assumed only that the ionisation of $\text{NH}$ (Equation 1) is fast compared to Reactions 2 and 3 in either direction.

Define: $\text{N}_T = \text{NH} + \text{N}\text{H}^+$ at any time; $\text{N}_T^\circ = \text{N}_T$ at $t = 0$

\[
K = \frac{\text{N}\text{H}^+}{\text{NH}}
\]

Since $K = \langle \text{NH} \rangle / \langle \text{NH}_\text{H}^- \rangle$,

\[
\text{N} = \alpha \text{N}_T
\]

\[
\text{NH} = (1 - \alpha)\text{N}_T
\]

\[
- \frac{d\text{N}_T}{dt} = k_1(1 - \alpha)\text{N}_T\text{A} + k_2\text{N}_T\text{A} - (k_3\text{OH}^- + k_4)\text{N}_T^\circ
\]

Let $k = k_3\text{OH}^- + k_4$

\[
k_{s\text{up}} = k_1(1 - \alpha) + k_2 + \frac{k}{A}
\]

Substituting in Equation 7,

\[
- \frac{d\text{N}_T}{dt} = k_{s\text{up}}\text{N}_T\text{A} - k\text{N}_T^\circ
\]

Since at equilibrium, $k_{s\text{up}}\text{N}_T\text{A} = k\text{N}_T^\circ$

\[
- \frac{d\text{N}_T}{dt} = k_{s\text{up}}\text{N}_T\text{A} - k\text{N}_T^\circ
\]

Integrating and setting $\text{N}_T = \text{N}_T^\circ$ at $t = 0$

\[
\ln \frac{\text{N}_T^\circ}{\text{N}_T^\circ - \text{N}_T} = -k_{s\text{up}}t
\]
or substituting from Equations 5 and 6,

\[
\ln \frac{N - N^*}{N^* - N} = \ln \frac{NH - NH^*}{NH^* - NH} = -k_{\text{app}} t
\]

Over the range of experimental conditions studied, \( k = k_0 \) for inosine and both pseudouridine isomers, while for 4-thiouridine, \( k = 0 \), although \( k_1 \) is a real number (see the text). Thus Equation 8 reduces to \( k = k_2(OH) \) for all examples studied leading to

\[
k_{\text{app}} - k_1 \frac{OH}{A} = k_2(1 - \alpha) + k_3 \alpha \tag{13}
\]

where \( \alpha \), by rearrangement of Equation 4, is given by

\[
pK - pH = \log \left( \frac{1}{\alpha} - 1 \right) \tag{14}
\]

### B. Absorbance as Measure of Concentration for Use in Equation 12

1. For any constant \( \lambda \) and \( pH \), absorbance \( (D) \) at time zero, \( t \), and equilibrium is given by

\[
D_0 = \varepsilon_{NH} N_H^o + \varepsilon_N N^o
\]

\[
D_t = \varepsilon_{NH} N_H + \varepsilon_N N^* + \varepsilon_{NH} N_AH
\]

\[
D_e = \varepsilon_{NH} N_H^e + \varepsilon_N N^e + \varepsilon_{NH} N_{AH}^e
\]

Substituting and subtracting

\[
D_t - D_e = \left( N^e - N^e \right) \left[ \varepsilon_{NH} (1 - \alpha) + \varepsilon_N \alpha - \varepsilon_{NH} \right]
\]

\[
D_e - D_0 = \left( N^o - N^o \right) \left[ \varepsilon_{NH} (1 - \alpha) + \varepsilon_N \alpha - \varepsilon_{NH} \right]
\]

Thus

\[
\frac{D_t - D_e}{D_e - D_0} = \frac{N^o - N^o}{N^o - N^o}
\]

and

\[
\log \frac{D_t - D_e}{D_e - D_0} = -\frac{k_{\text{app}} A}{2.303} t \tag{15}
\]

2. When \( k_3 = 0 \), \( D_e = D_0 \) and Equation 15 reduces to

\[
\log \frac{D_t - D_e}{D_0 - D_0} = -\frac{k_{\text{app}} A}{2.303} t \tag{16}
\]

where

\[
k_{\text{app}} = k_1 (1 - \alpha) + k_3 \alpha \tag{17}
\]

3. The same Relations 15 and 16 hold in cases in which the spectrum of the product \( N_AH \) changes as a function of \( pH \) because of an ionization elsewhere in the nucleotide. For example, this is the case with pseudouridines C and B, which after cyanoethylation still have a \( pK \) around \( pH \) 9.2 that produces spectral changes with \( pH \) (Fig. 10). The spectral changes do not affect Equations 15 and 16 because the quantity \( \varepsilon_{NH} \) in such cases is simply replaced by another constant representing the weighted contribution from both the ionized and un-ionized species at any \( pH \) and cancels out in Equation 15.
The Function of Pseudouridylic Acid in Transfer Ribonucleic Acid: I. THE
SPECIFIC CYANOETHYLATION OF PSEUDOUREDINE, INOSINE, AND
4-THIOUREDINE BY ACRYLONITRILE
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