A CHEMICAL METHOD FOR THE SELECTIVE MODIFICATION OF PSEUDOURIDINE IN THE PRESENCE OF OTHER NUCLEOSIDES*

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Pseudouridine (ψ), the 5-ribosyl analog of uridine, has been known for a number of years and its structure established as one in which the glycosyl linkage is C-C rather than the usual C-N (1). Pseudouridylic acid (ψP) is found in RNA, and with the recognition of the existence of a separate class of transfer RNA molecules, it has become clear that most, if not all, of the ψP found in RNA is localized in this fraction. Messenger and viral RNAs have so far not been found to contain any ψP, and except for one report describing appreciable quantities of ψP in wheat germ ribosomal RNA (2), little or no ψP exists in purified ribosomal RNAs. In view of the highly specific localization of this unusual nucleoside in an RNA fraction which plays a crucial role in the translation of genetic information, it has been tempting to speculate that ψP plays some role in the proper functioning of transfer RNA.

In order to examine this question, we have developed a specific chemical method for the modification of ψ, using conditions such that negligible reaction takes place with other nucleosides. Moreover, the reaction conditions are sufficiently mild to allow their application to ψ

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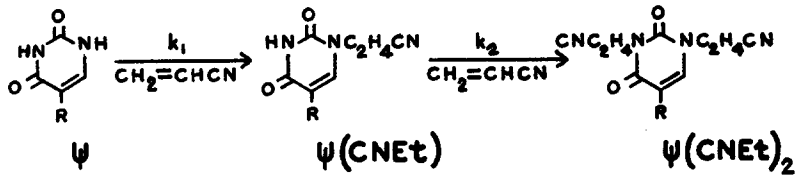
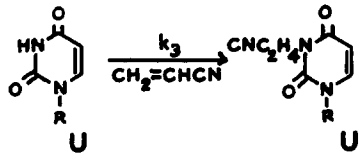
residues in intact transfer RNA.

The reaction is a Michael-type condensation of acrylonitrile with the ring nitrogens of ψ and uridine to form the N-cyanoethyl derivatives as shown in equations (1) and (2).



(1)



(2)

The formation of N-cyanoethyl ψ from ψ and acrylonitrile under strongly alkaline conditions and high temperature had previously been shown by Chambers, et al. (3). The purpose of this communication is to demonstrate that the reaction can be made highly specific for ψ and that reaction conditions can be found which allow the application of this technique to ψ residues in intact transfer RNA.

Materials and Methods

Acrylonitrile reaction: The conversion of ψ and uridine to the N-cyanoethyl derivatives was accomplished as follows. Sodium carbonate buffer, ionic strength 0.05 at the appropriate pH, was prepared and acrylonitrile dissolved to a concentration of 1 M. One-tenth volume of 0.1 M uridine or ψ was added and reaction allowed to take place at 30° in tightly stoppered tubes. Aliquots were removed as a function of time and the reaction terminated by neutralization and freezing.

Quantitation of the reaction: 10 µl. samples were chromatographed in Solvent A (Table I). After location of the samples under UV light, they were

Table I

Thin Layer Chromatography of Nucleosides and Their Derivatives

	R_U in Solvent			
Compound	A	B	C[a]	D
Pseudouridine	0.79	0.70	0.53	0.93
ψ(CNEt)	0.93	0.92	0.70	1.08
$\psi(CNEt)_2$	1.18	1.30	1.23	1.76
U(CNEt)	1.19	1.43	1.54	1.81
thymidine	1.23	1.38		
thymidine-X	1.37	1.66		
adenosine	0.97	1.13		
adenosine-X	1.16	1.39		
cytidine	0.82	0.80		
cytidine-X	1.05	1.09		
guanosine	0.71	0.73		
guanosine-X	0.98	1.03		
uridine (R_f value)	0.68	0.43	0.48	0.42

Solvent A, isopropanol-1% $(NH_4)_2SO_4$(2:1), Solvent B, n-butanol-acetic acid-water (5:1:4); Solvent C, isobutanol-water (88:12), Solvent D, isopropanol-NH_4OH-H_2O (7:1:2).

[a] Developed 3 times in this solvent.

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eluted and the amount of nucleoside determined by absorption spectrophotometry. The data were corrected for blank absorption and for elution efficiency as determined from a standard curve. An extinction coefficient, ϵ_{260}(pH 7) = 7470 (4) was used for ψ(CNEt) and $\psi(CNEt)_2$ as well as for ψ. Similarly, the value of ϵ_{260}(pH 7) = 9900 (5) for uridine was used also for U(CNEt). By analogy with the values obtained for the methylated uracils (6), it is not likely that the correct values will be different from those used here by more than 10%.

Purification of N-cyanoethyl derivatives: The derivatives were prepared by large scale incubations of the type described above, followed by chromatographic separation on 1 mm. cellulose layers in Solvent A. After elution of the desired bands, salts and any contaminants were removed by re-chromatography in Solvent B. These two steps were sufficient to completely purify ψ(CNEt) and $\psi(CNEt)_2$ but a further purification of U(CNEt) in Solvent D was necessary. The

derivatives were chromatographically homogeneous in all solvents tested (level of detection 1%) even when multiple development was employed.

Chromatography: Chromatographic separations were carried out on thin layers of cellulose at room temperature. The mobilities of the various compounds in the several solvent systems employed are summarized in Table I. Because of the well known variability of R_f values with thin layer techniques, the values are expressed as R_U, mobility relative to uridine.

Chemicals: Acrylonitrile, practical grade, was obtained from Eastman and re-distilled. It was stored at 5°. Pseudouridine, A grade, was purchased from Calbiochem and consisted of 69% C isomer, 29% B isomer, and 2% A_S isomer (7). The mixture of isomers was used. ψ5'P, B grade, was also obtained from Calbiochem. Its isomer composition was not determined.

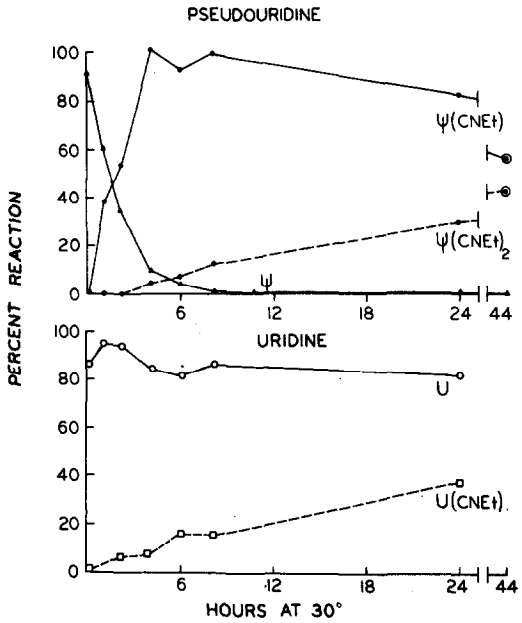


Fig. 1. Rate of conversion of ψ and uridine to their N-cyanoethyl derivatives. Reaction took place at pH 9.70 as described in the text. Quantitation of the amount of nucleoside formed or remaining was done as described in the text. The 44 hour samples were a separate experiment.

Results

Kinetics and specificity of the reaction: The result of incubation of ψ

and uridine with acrylonitrile at pH 9.70 is shown in Fig. 1. It is clear from the figure that at this pH there is a rapid conversion of ψ to ψ(CNEt), followed by a much slower appearance of $\psi(CNEt)_2$ which takes place at about the same rate as the conversion of uridine to U(CNEt). If the reaction is allowed to continue for 44 hours, there is a continued increase in the amount of $\psi(CNEt)_2$ formed with a concomitant decrease in the amount of ψ(CNEt), supporting the mechanism shown in eq. (1) in which ψ(CNEt) reacts with a second molecule of acrylonitrile to yield $\psi(CNEt)_2$.

Table II

Effect of pH on Reaction Rate of Uridine,
Pseudouridine and Thymidine with Acrylonitrile

	A				B			
pH	Apparent Rate Constant* (min^{-1}) x 10^3				True Rate Constant** (min^{-1}) x 10^3			
	ψ	U	T	ψ/U	ψ	U	T	ψ/U
9.15	4.57	0.24		19.	17.5	0.54		33.
9.60			0.18				0.48	
9.70	8.97	0.50		18.	16.1	0.68		24.
10.05	15.40	0.84		18.3	20.9	0.97		21.
				ave	18.2	0.73		25.

* Calculated from the slope of the line relating log % reaction vs. time for a pseudo first order reaction.

** k = (apparent rate constant)x(a); log (a-1) = pK_{ioniz} - pH where pK (pseudouridine) = 9.6 (8), pK (uridine) = 9.25 (6), pK (thymidine) = 9.8 (6).

Experimental conditions were as described for Fig. 1 except for variation in the pH of the reaction mixtures. The amount of reactant nucleoside present at any time was determined directly and also by calculation from the total products formed.

Consideration of the reaction mechanism (eq. 1) suggested that the rate of disappearance of ψ or uridine should be pseudo first order since acrylonitrile

was present in large excess. When the data of Fig. 1 were replotted on this basis, the apparent rate constants shown in Table IIA were obtained. It is clear that the reaction is highly specific for ψ, and that the rate of the reaction is strongly pH dependent. Such a strong pH dependence around the pK of ψ suggested the following mechanism (eq. 3)

$$\psi H \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} \psi^- + H^+ \xrightarrow[k_1]{\text{acrylonitrile}} \psi(CNEt) \qquad (3)$$

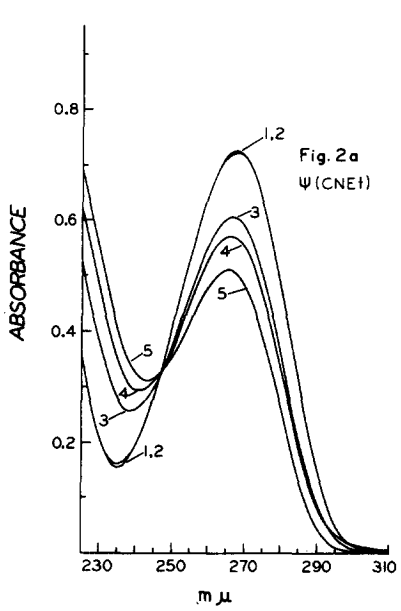
in which the anionic form of ψ is the reactive species. Calculation of the rate equation is considerably simplified by assuming that k_i and $k_{-i} \gg k_1$ and leads to the following relation (eq.4).

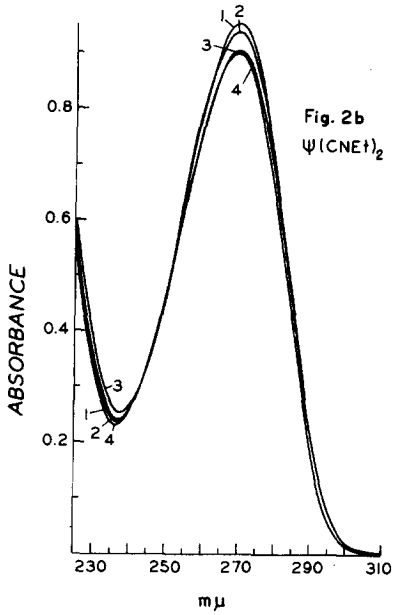
$$[\psi H + \psi^-] = [\psi H + \psi^-]_o e^{-Nk_1 t/a} \qquad (4)$$

where N = acrylonitrile concentration (moles/liter) and $\log(a-1) = pK_{ioniz} - pH$. When this correction is applied to the apparent rate constants, the results shown in Table IIB were obtained. The agreement obtained at different pH values is in support of the above mechanism.

The rate of the reaction with thymidine has also been determined (Table II). It is noteworthy that the slower apparent rate of reaction with thymidine compared to uridine can be accounted for on the basis of its higher pK value (compare IIA with IIB). Acrylonitrile reacts also with adenosine, guanosine and cytidine under these conditions to give a product with a higher mobility in Solvents A and B (Table I), but at a very much slower rate. Thus after 24 hours at pH 9.7, between 10 and 20% of the nucleosides had reacted, while after 4 hours, when essentially all of the ψ had disappeared, a barely detectable amount of product had formed (ca. 1-3%). Reaction takes place with ψ5'P in a similar manner as with ψ. Thus, in a preparative experiment, after 43 hours at pH 10.1, all of the ψ5'P had reacted, and about 65% had been further converted to the $\psi(CNEt)_2$5'P. Further studies on the rate of reaction of ψ5'P are now in progress.

Characterization of the product: The N-cyanoethyl derivatives have been characterized as such by (a) their relative kinetics of appearance (Fig. 1), (b) their relative mobility in the solvent systems employed (Table I), and (c)





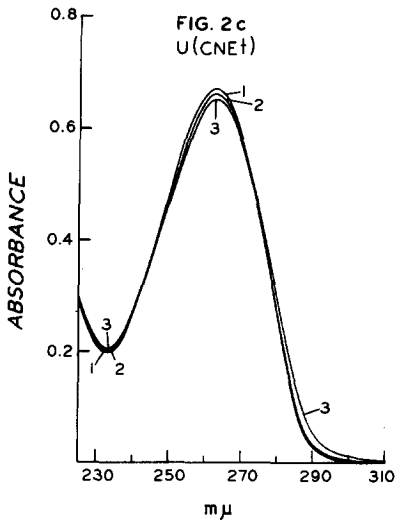


Fig. 2. Spectra of purified N-cyanoethyl derivatives as a function of pH. Curves were recorded on a Cary Model 14 Spectrophotometer and pH measurements made on the contents of the cuvettes with a Radiometer TTT1 Titrigraph equipped with scale expander.

(a) Ψ(CNEt). Separate dilutions were made in the appropriate buffers except where indicated and the spectra recorded versus the appropriate buffer. Curve 1, 0.05 M phosphate buffer pH 7.0; curve 2, solution of curve 1 acidified to pH 2.2; curve 3, sodium carbonate buffer pH 9.17; curve 4, sodium carbonate buffer pH 9.57, curve 5, 0.01 N NaOH. Calculated pK_a = 9.14.

(b) $\psi(CNEt)_2$. Spectra were recorded on a single sample whose pH was adjusted by addition of HCl or NaOH to both sample and reference cells. Curve 1, water solution pH 7.4. Curve 2, acidified to 0.01 N HCl. Curve 3, adjusted to 0.01 N NaOH, read immediately after pH adjustment. The curve was identical after 20 minutes at 30°. Curve 4, re-acidified to 0.01 N HCl. Calculation of the A_m values corrected for dilution relative to that at pH 2 gave: 1, 1.010; 2, 1.000; 3, 0.985; 4, 1.000.

(c) U(CNEt). Spectra were recorded as in (b). Curve 1, H_2O solution pH 6.8; curve 2, acidified to 0.01 N HCl; curve 3, adjusted to 0.01 N NaOH. Calculation of A_m values corrected for dilution relative to that at pH 2 gave: 1, 1.000; 2, 1.000; 3, 1.003.

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their spectral properties (Fig. 2). ψ, which has both an N_1 and N_3 nitrogen atom available for reaction yields two products, one rapidly and the second slowly, while uridine with only an N_3 nitrogen atom gives but one product at a rate similar to that for the second product of the ψ reaction. This suggests that the first product of the ψ reaction was an addition to the N_1 nitrogen, while the second product was an addition to the available N_3 nitrogen of the first product.

The chromatographic behavior of the products in all of the solvent systems employed show them to have an increased affinity for the organic phase, as expected for the addition of cyanoethyl groups. Moreover, the postulated $\psi(CNEt)_2$ shows the predicted increase in mobility over that of $\psi(CNEt)$. Formation of the carboxyethyl derivative by hydrolysis of the corresponding cyanoethyl compound during the course of the reaction would appear to be excluded on the basis of the R_ψ and R_U values for the purified compounds in Solvent D. Chambers, et al. (3) report an R_ψ of 0.72 (calculated R_U = 0.67) for N_1-carboxyethyl ψ, which could be readily detected if present. Similarly slower mobilities would be predicted for the other carboxyethyl derivatives since they would all possess additional charge in this solvent due to the carboxyl group dissociation.

The spectral characteristics of the products (Fig. 2) provide more convincing evidence for the proposed structures, particularly when compared with the spectra for the analogous N-methyl ψ derivatives described by Cohn (1). The relevant spectral properties can be summarized as follows:

1. Both ψ derivatives no longer show the bathochromic shift in alkali characteristic of a dissociable N_1-H (1).
2. ψ(CNEt) shows both a decrease of ε in alkali (A^{12}_{max}/A^{7}_{max} = 0.70) and an isosbestic point of 247 mμ. This behavior is characteristic of a dissociable N_3-H as shown by the similar behavior of N_1-methyl ψ(1), N_1-methyl uracil (6), thymidine, and uridine (9), all of which have A^{12}_{max}/A^{7}_{max} = 0.72 - 0.78 and isosbestic points in the range of 245 - 248 mμ.
3. $\psi(CNEt)_2$ shows no spectral changes over the pH range 2-12. This is indicative of the absence of both the N_3-H and the N_1-H as evidenced by the same behavior for 1,3-dimethyl ψ(1), and 1,3-dimethyl uracil (9).
4. U(CNEt) also shows no spectral changes over the pH range 2-12. This is to be expected for an N-cyanoethyl derivative of uridine which would also lack both the N_3-H and the N_1-H.

To summarize, the kinetic data and spectral evidence indicate addition to the N_1 and N_3 nitrogen atoms of the uracil ring and the chromatographic behavior supports the formulation of the products as the cyanoethyl rather than the carboxyethyl derivatives. Further characterization of the reaction products is in progress and will be reported subsequently.

Application to transfer RNA: Preliminary experiments on the extension of this approach to modification of ψ residues in intact transfer RNA (tRNA) have shown that the method is suitably mild, and does indeed lead to a marked alteration in the ability of tRNA to accept amino acids. Thus, after one hour at 60°, 87% of the acceptor activity of an acrylonitrile-treated sample of tRNA was lost while the control sample had lost only 7% of its activity. The products of the reaction have not been identified, however, nor is it certain that the loss in activity is due to specific modification of the ψ residues. In view of the nucleotide specificity demonstrated above, it is not likely that reaction with adenosine, guanosine, uridine, cytidine, or ribothymidine residues had caused the loss of function, but reaction with other minor components, such as the methylated bases cannot be excluded. Moreover, the possibility of modification of the observed reaction rates by unknown steric factors present in tRNA

cannot be excluded.

Experiments are underway to clarify these points and to unequivocally identify the reaction products of tRNA and acrylonitrile.

Acknowledgement

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