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# The Chemistry of Pseudouridine. IV. Cyanoethylation*

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ABSTRACT: Acrylonitrile reacts with adenosine, guanosine, uridine, cytidine, and pseudouridine at *p*H 11.5. However, at *p*H 8.8 selective cyanoethylation of pseudouridine is possible. The product is 1-cyanoethylpseudouridine. The mono- and dicyanoethyl derivatives of pseudouridine, pseudouridine-2′(3′)-phosphate, and pseudouridine-2′,3′-cyclicphosphate have been prepared. RNAase is active on 1-cyanoethylpseudouridinecyclicphosphate, but not on the 1,3-dicyanoethyl derivative. Selective cyanoethylation of uracil to give 1-cyanoethyluracil is described. Benzoylation of uracil to give a mixture of 1- and 3-benzoyluracil is also reported.

During the course of our studies on the chemistry of pseudouridine we observed that acrylonitrile reacted with the pyrimidine ring to give a monocyanoethyl derivative (Chambers *et al.*, 1963). The most remarkable feature of this reaction was that it appeared to be specific for the 1 position of pseudouridine. If cyanoethylation were really specific for pseudouridine and if this specificity could be maintained under mild conditions, then this reaction offered a potential route for selective modification of the pseudouridine residues in s-RNA. Therefore, we studied the cyanoethylation reaction in more detail.

In aqueous ethanol at *p*H 7 there was no detectable reaction between acrylonitrile and pseudouridine after 24 hours at room temperature. In dilute ammonium hydroxide (*p*H 8) a single new product was formed, but the reaction was slow and even after 64 hours 28% pseudouridine remained (Table I). Addition of $NH_4OH$ and a further 24-hour incubation gave only a slight increase in yield. Additions of both $NH_4OH$ and acrylonitrile finally brought the reaction near completion (Table I).

These results demonstrated that the reaction could be run under mild conditions. They also indicated that it was *p*H dependent and that ammonia was reacting with acrylonitrile. In order to inhibit this side reaction, the ammonium hydroxide was replaced by triethylammonium acetate buffer and the *p*H was raised to 8.8. Under these conditions the formation of monocyanoethylpseudouridine was rapid and after 3 hours the reaction was essentially complete (Figure 1). During this time no dicyanoethylpseudouridine could be detected. However, after 30 hours some of the disubstituted product had formed. Adenosine, guano-

TABLE I: Cyanoethylation of Pseudouridine.

| Time (hr) | Additions[a] | Ψ[b] |
|---|---|---|
| 0–64 | None | 28 |
| 64–88 | $NH_4OH$ | 23 |
| 88–96 | $NH_4OH$ + acrylonitrile | 15 |
| 96–120 | $NH_4OH$ + acrylonitrile | 5 |

[a] The reaction was carried out in dilute $NH_4OH$ at *p*H 8 with a 10-fold excess of acrylonitrile. Further additions were made as indicated (see Experimental for details). [b] Ψ = pseudouridine. Values are per cent of starting material remaining.

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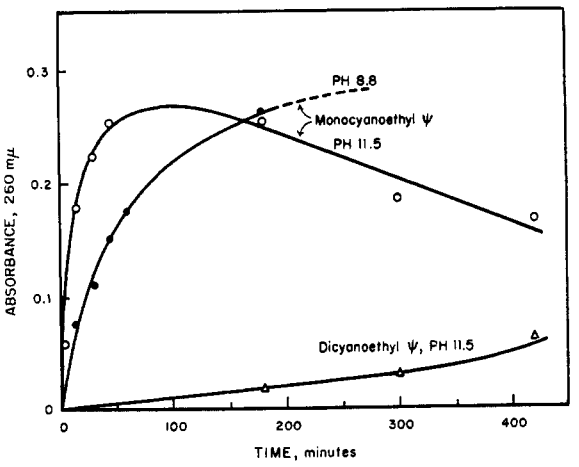


FIGURE 1: Cyanoethylation of pseudouridine. The absorbance values were obtained by quantitative paper chromatography and spectrophotometric analysis (see Experimental) and are proportional to yield. Ψ = pseudouridine.

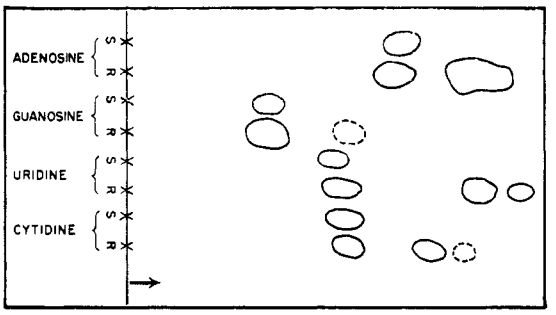


FIGURE 2: Cyanoethylation of adenosine, guanosine, uridine, and cytidine at *p*H 8.8 for 16 hours. This tracing is from a thin-layer plate run in solvent I. R = the individual reaction mixtures; S = the appropriate standard nucleosides. The dotted circles indicate trace components.

sine, uridine, and cytidine did not react in 3 hours under these conditions.

In order to examine the effect of *p*H further, the foregoing reactions were repeated at *p*H 11.5. As shown in Figure 1, the formation of cyanoethylpseudouridine was extremely fast. After 1 hour the starting material had disappeared and the major product was monocyanoethylpseudouridine. Only a trace of dicyanoethylpseudouridine was formed during this time, but as the reaction time was extended the amount of disubstituted derivative increased at the expense of monocyanoethylpseudouridine (Figure 1).

When the other ribonucleosides were tested at *p*H 11.5, only adenosine reacted in 1 hour, but after 16 hours the chromatographic pattern shown in Figure 2 was obtained. The structure of these new compounds has not been determined.

It is clear from these results that there is no absolute

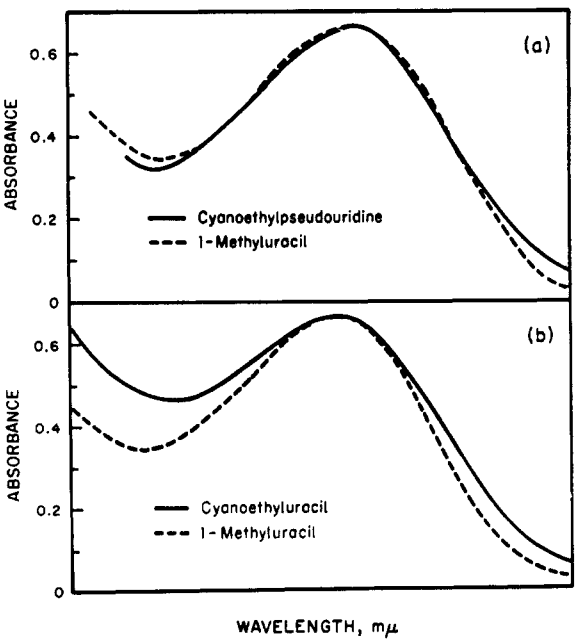


FIGURE 3: Ultraviolet absorption spectra of cyanoethylpseudouridine and cyanoethyluracil compared to 1-methyluracil. The 1-methyluracil spectrum (Shugar and Fox, 1952) was corrected so that its $\lambda_{max}$ and $\epsilon_{max}$ coincided with $\lambda_{max}$ and $\epsilon_{max}$ of cyanoethyluracil or cyanoethylpseudouridine.

specificity in the cyanoethylation reaction. Thus adenosine, guanosine, cytidine, uridine, and pseudouridine all react to some extent with acrylonitrile at high *p*H. However, the *rates* of the reactions at *p*H 8.8 are sufficiently different so that selectivity can be achieved by the proper choice of conditions. Thus it is possible to prepare monocyanoethylpseudouridine without any detectable reaction with other nucleosides.

The most interesting feature of this reaction is the selectivity for the 1 position of pseudouridine. The data presented previously (Chambers *et al.*, 1963) leave no doubt that the product is 1-cyanoethylpseudouridine. However, the possibility remained that a small amount of the 3-substituted derivative was present and escaped detection in our spectral and chromatographic studies. In order to test this we compared the *p*H 12 spectrum of 1-methyluracil (Shugar and Fox, 1952) with that of monocyanoethylpseudouridine (Figure 3a).

If we assume that the spectrum of 1-methyluracil, a well-characterized compound (Brown *et al.*, 1955), has the same shape as 1-cyanoethylpseudouridine then the spectra of these two compounds should be superimposable after suitable correction for differences in $\epsilon_{max}$ and $\lambda_{max}$. As can be seen in Figure 3a, the agreement is fairly good.[1]

[1] The same argument would hold using the more closely related 1-methylpseudouridine (Cohn, 1960), and the curves fit well. However, this derivative is less well characterized than 1-methyluracil.

3-Methylpseudouridine (Cohn, 1960) and 3-methyluracil (Shugar and Fox, 1952) have a $\lambda_{max}$ in the 285-m$\mu$ region at *p*H 12. 3-Cyanoethylpseudouridine should show a similar $\lambda_{max}$. Therefore, the presence of this isomer would be reflected by a hyperchromicity of the cyanoethylpseudouridine spectrum compared to that of 1-methyluracil around 285 m$\mu$. As shown in Figure 3a, the curve for cyanoethylpseudouridine *is* above that of 1-methyluracil in this region. However, the difference is small and it is not possible to decide definitely whether this represents the presence of a small amount of 3-cyanoethylpseudouridine or simply an inherent difference in the spectra of these two compounds. It is clear that the formation of the 1 isomer is greatly if not exclusively favored over the 3 isomer.

The reasons for the preferential reaction of acrylonitrile with the 1 position of pseudouridine are not entirely clear, but a partial explanation can be constructed. The increase in reaction rate that occurs as the *p*H is increased suggests that an ionized form of pseudouridine is the reactive species. In the *p*H range studied pseudouridine exists as a mixture of monoanions (Figure 4, R = $\beta$-D-ribosyl) in which the 1 anion (II)[2] predominates over the 3 anion (III) by a ratio of about 4:1.[3] Therefore, other things being equal, the distribution of monoanions favors the formation of the 1-cyanoethyl derivative.

If this were the only factor operating then one would predict that cyanoethylation of uracil at *p*H 8.8 should produce a mixture of the 1- and 3-cyanoethyl derivatives because uracil shows an *equal* distribution of its tautomeric monoanions (Figure 4, R = H) (Nakanishi *et al.*, 1961). However, when this reaction was carried out, only a single monocyanoethyluracil could be detected by paper chromatography. The ultraviolet spectrum of this product at *p*H 12 (Figure 3b) shows that it is mainly 1-cyanoethyluracil.[4]

Again, a small contribution by the 3-substituted isomer cannot be ruled out because the cyanoethyluracil spectrum shows a slightly higher absorbance at 285 m$\mu$ than does 1-methyluracil. However, this difference may also be due to the inherent spectral properties of the two compounds and not to the presence of the

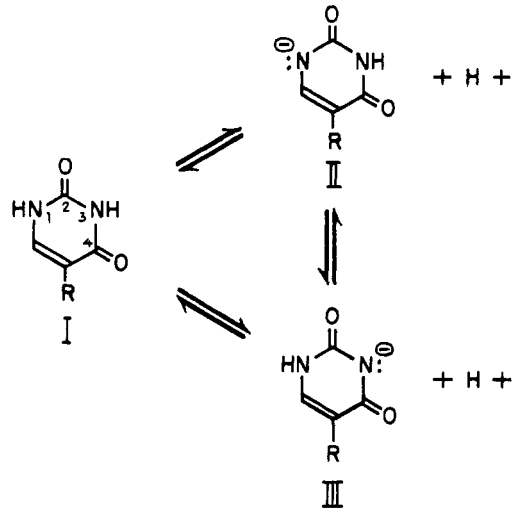


FIGURE 4: The tautomeric uracil monoanions.[2]

3 isomer. In any case, it appears that the *1 anion is inherently more reactive than the 3 anion* and that *preferential cyanoethylation occurs at the 1 position regardless of the tautomer distribution.*

As far as we are aware, the reactions described here provide the first examples of direct, *specific* alkylation of the ring nitrogen of uracil or pseudouridine. A closely related reaction of thymine with chloroacetic acid at *p*H 10 has been reported (Rabinowitz and Gurin, 1953), but no data to support the structural assignment were given. However, Dr. Jack Fox (personal communication) has repeated this reaction and has concluded from spectral data that the product is indeed thymine-1-acetic acid.

Considerable care must be exercised in generalizing these results. For example, it has been reported (Scannell *et al.*, 1959) that methylation of uracil or pseudouridine with dimethyl sulfate at *p*H 9 gives a mixture consisting of about equal amounts of the 1- and 3-methyl derivatives. Furthermore, thymine can be methylated under these conditions to give 1,3-dimethylthymine, but 6-methylthymine gives *only* 3-methylthymine. Methylation of pseudouridine with diazomethane in neutral solution also produces a mixture of products (Cohn, 1960; R. W. Chambers, unpublished experiments). We have found that monobenzoyluracil formed by reacting benzoyl chloride with uracil in pyridine is clearly a mixture of 1- and 3-benzoyluracil as shown by the distinct maxima at 257 and 283.5 m$\mu$ in the ultraviolet spectrum at *p*H 12 (Figure 5). However, it has been claimed (Spector and Keller, 1958) that acetylation of uracil with refluxing acetic anhydride gives 1-acetyluracil exclusively. Thus the course of the reaction appears to be influenced by the structure of the starting material (including its ionic form) *and* the nature of the reagent employed.

With a method of selective modification of pseudouridine at hand it was of interest to see if this specificity held at the nucleotide level and what effect the cyano-

[2] Spectral evidence suggests that the 1 anion of uracil (II, R = H) actually exists with the charge on the oxygen atom (Nakanishi *et al.*, 1961). The same is probably true of pseudouridine, but for simplicity both anions have been described in the same way.

[3] This can be deduced from the spectra of pseudouridine and 1- or 3-methylpseudouridine at *p*H 12, as pointed out first for uracil by Nakanishi *et al.* (1961). Tautomerism of pseudouridine will be the subject of a separate communication from this laboratory.

[4] The spectra of 2- and 4-ethoxyuracil and 3-methyluracil (Shugar and Fox, 1952) all show bathochromic shifts, to varying degrees, at *p*H 12 compared to *p*H 7. Cyanoethyluracil, on the other hand, like 1-methyluracil, shows a slight hyposochromic shift. This combined with a decrease in $\epsilon_{min}$ and a red shift of $\lambda_{min}$ at *p*H 12, compared to *p*H 7, establishes the structure as 1-cyanoethyluracil. The *p*H 7 spectrum of 1-cyanoethyluracil is not reproduced here since it is similar to the well-known spectrum of uracil itself at *p*H 7.

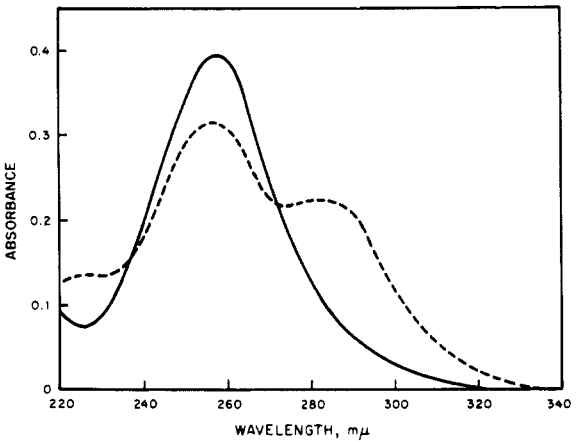


FIGURE 5: The ultraviolet absorption spectrum of monobenzoyluracil. ———, *p*H 6; ---------, *p*H 11.5.

Dekker, 1960). According to this theory the hydrogen on N-3 and the adjacent 4-carbonyl of pseudouridine participate in the hydrolysis of the diester as shown in Figure 6. Since the hydrogen on N-1 is not involved directly, this mechanism predicts, as found, that substitution of N-1 would have no effect electronically on RNAase action.[5] It would be particularly interesting to test the action of RNAase on a 3-substituted pseudouridine cyclicphosphate because it can be seen from Figure 6 (dotted arrows) that such a compound can undergo electronic shifts similar to those of the 1-substituted derivative. However, the stereorelationship between these two N—H groups and the participating carbonyl at C-4 is quite different. Unfortunately, no derivative suitable for testing this prediction has been prepared to date. It should also be emphasized that these findings are equally compatible with other theories of RNAase action (e.g., Findlay *et al.*, 1961).

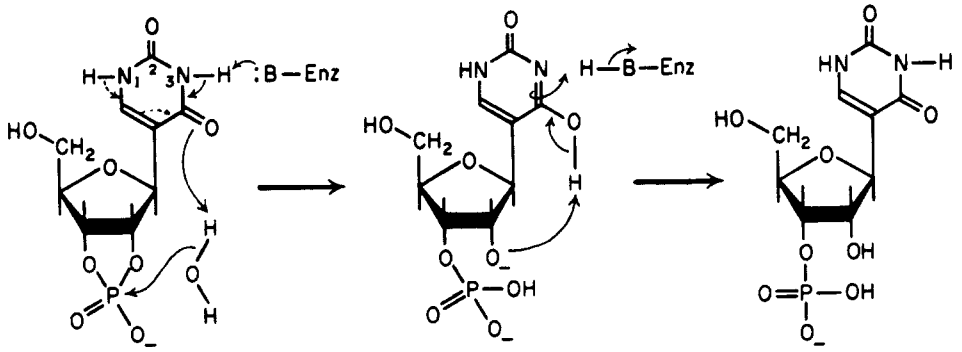


FIGURE 6: RNAase-catalyzed hydrolysis of pseudouridine-2′,3′-cyclicphosphate according to the Witzel (1960)–Dekker (1960) hypotheses.

ethyl group would have on the action of ribonuclease. Accordingly, the reaction was repeated on the mixed 2′(3′)-mononucleotides. The results were similar to those found with the nucleosides. Thus, it was possible to form 1-cyanoethylpseudouridine-2′(3′)-phosphate under conditions where adenylic, guanylic, uridylic, and cytidylic acids did not react. Conditions have *not* been found so far where dicyanoethylpseudouridylic acid can be prepared without reaction of acrylonitrile with adenylic, guanylic, and uridylic acids (cytidylic acid did not react under the conditions studied).

In order to see if the presence of cyanoethyl groups had any effect on RNAase activity, 1-cyanoethylpseudouridine-2′(3′)-phosphate and 1,3-dicyanoethylpseudouridine-2′(3′)-phosphate were converted to their corresponding cyclicphosphates. 1-Cyanoethylpseudouridine-2′,3′-cyclicphosphate was cleaved rapidly to 1-cyanoethylpseudouridine monophosphate (presumably the 3′ isomer) by RNAase. 1,3-Dicyanoethylpseudouridine-2′,3′-cyclicphosphate was unaffected even after several days' incubation with the enzyme.

These results with RNAase agree with the Witzel-Dekker hypothesis of RNAase action (Witzel, 1960;

It was disappointing, but not surprising, that selective cyanoethylation in the 1 position had no effect on RNAase action. This precludes the use of the reaction to prevent RNAase-catalyzed hydrolysis at $\Psi_pX$ ($\Psi$ = pseudouridine, X = any other nucleoside) bonds in s-RNA. It would still be of interest to see if the specificity of the reaction can be maintained at the polynucleotide level and what effect it has on the biological activity of s-RNA.

## Experimental

*Analytical Methods.* Paper chromatography was carried out by the descending technique on Whatman No. 40 paper. Quantitative analysis was performed by spotting 2-μl aliquots. After the chromatograms were developed the ultraviolet-absorbing materials were

[5] In uridine, the sugar is attached to N-1. The hydrogen on N-3 and the *2-carbonyl* are postulated as the participating groups in this case (Dekker, 1960). The importance of the N-3 hydrogen is supported by the work of Szer and Shugar (1961), who found that 3-methylpolyuridylic acid and 3-methyluridine-2′,3′-cyclicphosphate are *not* substrates for RNAase.

located with an ultraviolet lamp (Mineralite, Ultraviolet Products, South Pasadena, Calif.) equipped with a short wavelength filter (Model SL 2537). The material was eluted with 3.0 ml of 0.02 M sodium phosphate buffer, *p*H 7.0, and the absorbance was measured against appropriate paper blanks in a Beckman DU spectrophotometer.

Thin-layer chromatography was carried out on 0.25-mm-thick layers of cellulose (MN-cellulose powder 300 without binder, Brinkman Instruments, Great Neck, N.Y.) on plate glass. The solvent was allowed to ascend 10 cm. The ultraviolet-absorbing material was located by holding the plate, *cellulose layer down*, over the Mineralite. The spots, which were visible through the glass, were outlined on the glass with a marking pen. Tracings were made from this for permanent records.

The solvent systems employed were: (I) 2-propanol–concd. ammonium hydroxide–water (7:1:2); (II) 95% ethanol–0.1 M ammonium acetate, *p*H 7 (7.5:3); (III) 1-propanol–concd. ammonium hydroxide–water (11:7:2). The $R_F$ values are given in Table II.

TABLE II: $R_F$ Values[a]

| Compound | Solvent | | |
|---|---|---|---|
| | I | II | III |
| Pseudouridine | 0.33 | | |
| 1-Cyanoethylpseudouridine | 0.46 | | |
| Dicyanoethylpseudouridine | 0.64 | | |
| Pseudouridine-2′-phosphate | 0.06* | 0.10 | 0.24* |
| Pseudouridine-2′,3′-cyclic-phosphate | 0.28 | | |
| 1-Cyanoethylpseudouridine-2′-phosphate | 0.13 | 0.23* | 0.29 |
| | 0.11* | | 0.22* |
| Dicyanoethylpseudouridine-2′-phosphate | 0.32* | | 0.47 |
| | | | 0.39* |
| 1-Cyanoethylpseudouridine-2′,3′-cyclicphosphate | 0.44 | 0.67* | |
| Dicyanoethylpseudouridine-2′,3′-cyclicphosphate | 0.61 | | 0.68 |

[a] These values are for paper chromatography, except those marked with an asterisk (*), which are for thin-layer chromatography.

Paper electrophoresis was carried out in an E–C apparatus (E–C Apparatus Co., Swathmore, Pa.) on Whatman 3MM paper. Ultraviolet spectra were obtained with a Beckman DK-2 recording spectrophotometer and the curves were replotted using a linear wavelength scale. Spectral constants[6] were obtained from measurements made with a Beckman DU spectrophotometer in 1-cm quartz cells. The values are regarded as accurate to ±2%. Infrared spectra were obtained with a Perkin-Elmer 237B spectrophotometer using KBr disks.

*Synthesis of Monobenzoyluracil.* This preparation was carried out in 1960 by Mr. Bernard Moss. Dry uracil (5.4 g) was suspended in 250 ml of anhydrous pyridine. Benzoyl chloride (71 ml) was added and the mixture was shaken mechanically for 86 hours. The uracil dissolved soon after the reaction was started. The clear solution gradually became cloudy and a tan solid separated. At the end of the reaction the solution was cherry red. The solid (pyridine hydrochloride) was removed by filtration and dried over $P_2O_5$, 3.55 g. The filtrate was poured rapidly onto cracked ice. The water was decanted from the dark-red oil. The latter was mixed with chloroform and extracted twice with water. The aqueous layers were combined and extracted twice with chloroform. The chloroform layers were combined and evaporated under reduced pressure. When the volume reached about 100 ml, crystals began to form. Evaporation was interrupted and small portions of ethyl acetate were added at 0° to complete crystallization. The product was collected by filtration, washed with ethyl acetate, and dried *in vacuo* at room temperature over $P_2O_5$, 4.436 g. A second crop was obtained by further evaporation of the mother liquor, 1.930 g; total yield 6.37 g. The product was homogeneous by paper chromatography in 2-propanol–1% $(NH_4)_2SO_4$ (2:1); mp 203–206° (uncorr).

*Anal.*[7] Calcd for $C_{11}H_8N_2O_5$ (216.19): C, 61.11; H, 3.73; N, 12.96. Found: C, 61.20; H, 3.65; N, 13.11. Spectral constants[8]: $\lambda^7_{max}$ 257 mμ; $\alpha^7_{257}$ 12.0 × $10^3$; $\lambda^7_{min}$ 225 mμ; $\alpha^7_{225}$ 3.3 × $10^3$; $\lambda^{11.5}_{max}$ 283.5, 267 mμ; $\alpha^{11.5}_{283}$ 6.2 × $10^3$, $\alpha^{11.5}_{267}$ 9.0 × $10^3$; $\lambda^{11.5}_{min}$ 225–230 mμ; $\alpha^{11.5}_{227}$ 4.4 × $10^3$.

*1-Cyanoethyluracil.* A triethylammonium acetate buffer was prepared by adjusting a mixture of water (15 ml), absolute ethanol (15 ml), and triethylamine (12 ml) with glacial acetic acid to *p*H 8.75. Uracil (224 mg, 2 mmoles) was suspended in 30 ml of the buffer and acrylonitrile (2.5 ml) was added. The mixture was shaken mechanically until a clear solution was obtained (2 hours). Thin-layer chromatography (solvent I) indicated that partial conversion of uracil to monocyanoethyluracil had occurred. After 4 hours the reaction was about 50% complete; after 6 hours, 70%.

[6] Superscripts refer to *p*H; subscripts to wavelength in mμ.

[7] Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.

[8] The α refers to molar absorbance. It must be emphasized that these values represent the actual absorbance obtained for a 1 M solution of benzoyluracil at the wavelength and *p*H indicated. Since benzoyluracil is a mixture of the 1- and 3-benzoyl derivatives, these α values are *not* to be confused with the *molar extinction* coefficients, ε, of the pure compounds. The values of ε are unknown although they can be estimated from the spectral data. The superscripts refer to *p*H. The *p*H 11.5 data were obtained within 2 minutes after adjusting the *p*H to 12. The constants were unchanged after 8 minutes at *p*H 12. This rules out the possibility that the peak at $\lambda_{283}$ was caused by hydrolysis of the acyl group under alkaline conditions.

After 7 hours, washed Dowex 50 ($H^+$) ion-exchange resin was added with stirring until the *p*H dropped to 6.9. The resin was removed by filtration and washed with 95% ethanol. Evaporation of the filtrate and washings gave a crystalline solid. The crystals were removed by filtration, washed with cold water, and recrystallized from hot water. The product was collected and dried over $P_2O_5$ *in vacuo* at room temperature, 200 mg. It gave a single component in solvent I (paper chromatography). Its melting behavior was peculiar. It softened at 214–219° and gave off a vapor which collected on the wall of the capillary at 220°, and then the residual solid melted at 228.5–230° (uncorr).

*Anal.*[7] Calcd for $C_7H_7N_3O_2$ (165.15): C, 50.90; H, 4.27; N, 25.44. Found: C, 51.16; H, 4.22; N, 25.27.

A sample of this material was dissolved with difficulty in aqueous ethanol and aliquots were diluted with either water or 0.05 M $K_2HPO_4$ buffer, *p*H 12.0, for measurement of the ultraviolet spectral constants: $\lambda_{max}^{6.4}$ 262, $\epsilon_{max}^{6.4}$ 9.4 × $10^3$; $\lambda_{min}^{6.4}$ 230, $\epsilon_{min}^{6.4}$ 1.6 × $10^3$; $\lambda_{max}^{12}$ 262, $\epsilon_{max}^{12}$ 7.1 × $10^3$; $\lambda_{min}^{12}$ 241, $\epsilon_{min}^{12}$ 4.3 × $10^3$. The infrared spectrum resembled that of 1-methyluracil. In addition, it displayed the characteristic C≡N stretching frequency at 2255 $cm^{-1}$.

*1,3-Dicyanoethyluracil.* Uracil (224 mg, 2 mmoles) was suspended in 5 ml $H_2O$ + 5 ml 95% ethanol. When 2 ml of triethylamine was added the solid dissolved. Acrylonitrile (2.5 ml) was added and the reaction was followed by removal of aliquots from the homogeneous reaction mixture for thin-layer chromatography in solvent I. After 19 hours uracil was no longer detectable; the products were dicyanoethyluracil (~80%) and monocyanoethyluracil (~20%). After 43 hours only dicyanoethyluracil was detectable. The mixture was evaporated to an oil and dried to a powder by addition and azeotropic distillation of absolute ethanol under reduced pressure. The solubility of the product was unusual. It was soluble in water, ethanol, ethyl acetate, acetone, dioxane, chloroform, and benzene, but insoluble in ether. The crude product was crystallized from dioxane by addition of ether, 218 mg. A sample was recrystallized from the same system and dried over $P_2O_5$ *in vacuo* at room temperature for analysis; mp 78–79°.

*Anal.*[7] Calcd. for $C_{10}H_{10}N_4O_2$ (218.21): C, 55.04; H, 4.62; N, 25.68. Found: C, 55.11; H, 4.65; N, 25.66. Spectral constants were obtained as described for 1-cyanoethyluracil: $\lambda_{max}^{6.4}$ 264, $\epsilon_{max}^{6.4}$ 9.1 × $10^3$; $\lambda_{min}^{6.4}$ 233, $\epsilon_{min}^{6.4}$ 1.8 × $10^3$; $\lambda_{max}^{12}$ 264, $\epsilon_{max}^{12}$ 9.1 × $10^3$; $\lambda_{min}^{12}$ 233, $\epsilon_{min}^{12}$ 1.8 × $10^3$. The infrared spectrum showed no N–H stretching in the 3000–3200 $cm^{-1}$ region nor the out-of-plane N—H bending at 900 $cm^{-1}$. It showed the typical carbonyl bands at 1711 and 1664 $cm^{-1}$ as well as the C≡N stretching at 2250 $cm^{-1}$.

*Cyanoethylation of Pseudouridine.* (A). WITH AMMONIUM HYDROXIDE. Pseudouridine (2.4 mg, 0.01 mmole) was dissolved in 0.2 ml of 0.05 M $NH_4OH$ and 0.05 ml of 95% ethanol. Acrylonitrile (7 μl, ~0.1 mmole) was added and the mixture (*p*H ~ 8) allowed to stand at room temperature. The reaction was followed by quantitative paper chromatography in solvent I. The results are shown in Table I. At the times indicated in Table I, 7 μl of acrylonitrile and/or 0.05 ml of 0.05 M $NH_4OH$ were added.

(B). WITH TRIETHYLAMMONIUM ACETATE, *p*H 8.8. Water (0.5 ml), absolute ethanol (0.5 ml), and triethylamine (0.2 ml) were mixed and adjusted to *p*H 8.8 with glacial acetic acid. Pseudouridine (2 mg) was dissolved in 0.12 ml of this buffer and 25 μl of acrylonitrile was added. The reaction was followed by quantitative paper chromatography in solvent I. Pseudouridine was converted to monocyanoethylpseudouridine (Chambers *et al.*, 1963) in 3 hours. There was no evidence of any other product. After 30 hours a new product, $R_F$ 0.64 (solvent I), was detected. Its ultraviolet spectra gave the following constants: $\lambda_{max}^{7}$ 268, $\lambda_{min}^{2}$ 236; $\lambda_{max}^{12}$ 268, $\lambda_{min}^{12}$ 238 mμ. This small shift may be caused by ionization of a sugar hydroxyl (Fox *et al.*, 1953). On the basis of this information the structure was assigned as 1,3-dicyanoethylpseudouridine.

(C). WITH TRIETHYLAMINE, *p*H 11.5. The reaction mixture contained pseudouridine (2 mg), water (0.05 ml), ethanol (0.05 ml), triethylamine (0.02 ml), and acrylonitrile (0.025 ml). The reaction was followed as before. After 1 hour, the only detectable product was monocyanoethylpseudouridine. Between 1 and 7 hours, both mono- and dicyanoethylpseudouridine were detected. Quantitative measurements were not carried beyond 7 hours, but after 16 hours the only detectable product was dicyanoethylpseudouridine.

*Cyanoethylation of Adenosine, Guanosine, Cytidine, and Uridine.* The reactions were performed on 2-mg samples of each of these nucleosides exactly as described in (B) and (C) above. Guanosine was not completely soluble in the *p*H 8.8 reaction mixture. The reactions were followed by thin-layer chromatography in solvent I. At *p*H 8.8 there was no detectable reaction with any of these nucleosides after 3 hours. The reactions were not followed further with time. At *p*H 11.5, only adenosine had reacted at all after 3 hours. After 16 hours, the pattern shown in Figure 2 was obtained. Note that guanosine showed only a trace of a new product.

*1-Cyanoethylpseudouridine-2′-phosphate.* Pseudouridine-2′-phosphate was prepared by Cohn's method (1961). A 1-ml aliquot taken from a stock solution containing 120 $A_{260}$/ml was concentrated to 0.15 ml. Ethanol (0.15 ml of 95%), triethylamine (0.06 ml), and acrylonitrile (0.075 ml) were added. The homogeneous reaction mixture was allowed to stand at room temperature. Thin-layer chromatography (solvent II) indicated the reaction was complete in 3 hours. The solution was evaporated to 0.1 ml and streaked on a 15-cm sheet of Whatman No. 40 paper. Chromatography in solvent III gave three bands, $R_F$ 0.24 (starting material), 0.29 (major product), and 0.47 (dicyanoethyl derivative). 1-Cyanoethylpseudouridine-2′-phosphate ($R_F$ 0.29 band) was eluted with water and characterized by its ultraviolet spectrum: $\lambda_{max}^{6}$ 267 mμ, $\lambda_{min}^{6}$ 235 mμ; $\lambda_{max}^{12}$ 263 mμ, $\lambda_{min}^{12}$ 245 mμ.

The dicyanoethylpseudouridine-2′-phosphate band

($R_F$ 0.47) was also eluted and characterized by its ultraviolet spectra. The *p*H 7 and 12 spectra were identical except for a small bathochromic shift of $\lambda_{min}$ from 236.5 m$\mu$ (*p*H 7) to 239.5 m$\mu$ (*p*H 12).

*Cyanoethylation of Adenylic, Guanylic, Cytidylic, and Uridylic Acids.* Samples (1.5 mg) of commercially available (Schwarz Bioresearch, Orangeburg, N.Y.) mixed 2',3'-mononucleotides were treated as described for cyanoethylation of pseudouridine-2'-phosphate. Adenylic and cytidylic acids were used as their free acids; uridylic and guanylic acids as their disodium salts. The reactions were followed by thin-layer chromatography in solvents I and III. After 16 hours there was no reaction with CMP. UMP ($R_F$ 0.18, solvent III) had partially reacted to give a single new product ($R_F$ 0.28, solvent I; 0.31, solvent III). This reaction was complete in 54 hours. CMP still had not reacted. AMP ($R_F$ 0.16, solvent III) had partially reacted after 16 hours to give a new product ($R_F$ 0.60, solvent III). GMP apparently reacted completely giving several fluorescent spots ($R_F$ 0.07, 0.18, 0.40, and 0.60, solvent III). These reactions were carried out before the importance of *p*H was fully appreciated. The *p*H was not measured, but it was probably between 10 and 11.

*Synthesis of Cyclicphosphates.* (A). PSEUDOURIDINE-2',3'-CYCLICPHOSPHATE. An aliquot (1 ml) from a stock solution of ammonium pseudouridine-2'-phosphate[9] (*vide supra*) was evaporated to dryness. The residue was taken up in formamide (0.02 ml) and ammonium hydroxide (0.02 ml of 2 N). Dicyclohexylcarbodiimide (10 mg) dissolved in *tert*-butyl alcohol (0.05 ml) was added and the heterogeneous solution was heated at 75–80° in a tightly stoppered flask. Thin-layer chromatography in solvent I indicated the reaction was complete in 3 hours. The mixture was cooled and the liquid was separated from the crystalline solid with a long-tipped dropper. The crystals were washed with several drops of water. The supernatant and washes were combined and the solution was evaporated to dryness under reduced pressure. The residue was taken up in a small amount of water and streaked on a 15-cm strip of Whatman No. 40 filter paper. Chromatography in solvent I gave three bands: $R_F$ 0.06 (starting material), 0.18 (amidate?), and 0.28 (cyclicphosphate, major product). All three bands were eluted with a small volume of water. Their behavior on paper electrophoresis and their ultraviolet spectra were consistent with the foregoing assignments. The presence of amidate and monophosphate was attributed to the presence of ammonia in solvent system I.

(B). 1-CYANOETHYLPSEUDOURIDINE-2',3'-CYCLICPHOSPHATE. 1-Cyanoethylpseudourine-2'-phosphate (76 $A_{260}$ units in 1 ml of water), described earlier, was converted to its cyclicphosphate exactly as described for pseudouridine-2'-phosphate. The product was isolated by paper chromatography in solvent I. The three bands were identified as 1-cyanoethylpseudouridine monosphosphate ($R_F$ 0.13), 1-cyanoethylpseudouridine-2'(3')-phosphoramidate ($R_F$ 0.32) (tentative), and 1-cyanoethylpseudouridine-2',3'-cyclicphosphate ($R_F$ 0.44).

(C). DICYANOETHYLPSEUDOURIDINE-2',3'-CYCLICPHOSPHATE. Pseudouridine-2'-phosphate was treated as described for cyanoethylpseudouridine except that 0.1 ml of acrylonitrile was used and the reaction was allowed to proceed for 5 days. Thin-layer chromatography showed that the starting material ($R_F$ 0.24, solvent III) had been completely converted to the disubstituted derivative ($R_F$ 0.39, solvent III; 0.32, solvent I).

The solvent was removed under reduced pressure. The residue was dissolved in ammonium hydroxide (0.05 ml of 2 N) and formamide (0.05 ml). Dicyclohexylcarbodiimide (25 mg) in 0.125 ml of *tert*-butyl alcohol was added. The remainder of the procedure was the same as described in (A). Isolation by paper chromatography in solvent I gave a major band at $R_F$ 0.61 and a minor one at $R_F$ 0.52 in solvent I. The $R_F$ 0.61 material was eluted with water. Its ultraviolet spectra at *p*H 7 and 12 were identical except for a small shift in $\lambda_{min}$ from 236.5 to 239.5 m$\mu$. On the basis of this spectral and chromatographic evidence the structure was assigned as 1,3-dicyanoethylpseudouridine-2',3'-cyclicphosphate.

*Hydrolysis of Cyclicphosphates by RNAase.* Aliquots (25 $\mu$l) of the aqueous solutions of the cyclicphosphates described were mixed with 5 $\mu$l of 0.1 M sodium acetate buffer, *p*H 5.6. A 10-$\mu$l aliquot of an RNAase solution (prepared from crystalline pancreatic RNAase, Worthington Biochemical Corp., Freehold, N.J., 10 mg/ml in the above-mentioned buffer) was added at 0°. Zero-time aliquots were immediately removed and spotted for thin-layer chromatography in solvent III. The mixtures were then incubated at 37° and the reaction was followed by thin-layer chromatography. Pseudouridine cyclicphosphate and its monocyanoethyl derivative were hydrolyzed completely to their corresponding monophosphates in 2–3 hours. Dicyanoethylpseudouridine cyclicphosphate was unchanged even after 3 days.

[9] A mixture of the 2' and 3' isomers will serve equally well as starting material.

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# Effects of Light in the Presence of Iron Salts on Ribonucleic Acid and Model Compounds*

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ABSTRACT: Under the influence of visible light in the presence of small amounts of ferric iron, tobacco mosaic virus ribonucleic acid becomes degraded and loses infectivity at 0° and neutral *p*H. Ferrous iron has the same catalytic effect at a somewhat lesser initial rate. The same reactions seem to proceed very much more slowly in the dark. Experiments with nucleotides and other model compounds show that bases are destroyed and released from glycosidic linkage under the influence of iron plus visible light. It appears probable that these are the primary events also with ribonucleic acid, and the diester bond breakage occurs secondarily. Hydrogen peroxide potentiates the photoeffects, particularly of $Fe^{2+}$, on both tobacco mosaic virus ribonucleic acid and model compounds, and catalase suppresses them.

Previous studies of the interaction of TMV-RNA[1] with certain metal ions have shown that some metal complexes were of decreased infectivity while others were fully infectious. Certain metals of both types rendered the RNA more enzyme resistant. Removal of the inactivating metals restored the original infectivity of the RNA (Singer and Fraenkel-Conrat, 1962).

Later studies in which radioactive metals were used led to the conclusion that alkali earth metals were bound primarily to the phosphate groups, while silver and mercury were bound each to a specific site on two of the four bases. Greater amounts of bound silver and mercury were displaced by trivalent metals ($In^{3+}$, $Al^{3+}$, $Fe^{3+}$), although the use of $^{59}Fe$ indicated that the RNA bound $Fe^{3+}$ less firmly than $Ag^{+}$, $Hg^{2+}$, or $Ca^{2+}$ (Singer, 1964).

The effect of iron salts on the infectivity of TMV-RNA differs from those of other metals in that iron causes progressive and irreversible inactivation. Upon exposure of the iron complexes ($Fe^{3+}$ and $Fe^{2+}$) to visible light this inactivation, which is not reversed by removal of the metal, proceeds very much more rapidly. The present report deals with the mechanism of this photosensitization of RNA by iron and the effect of EDTA and hydrogen peroxide on this system, as studied with both the intact RNA and its small molecular components.

## Methods and Materials

TMV-RNA was prepared as previously described (Fraenkel-Conrat *et al.*, 1961). $^{14}C$-Labeled TMV was isolated by the method of Sugiyama and Fraenkel-Conrat (1963). $FeCl_3$ and $FeSO_4$ were analytical grade reagents. Solutions of each in $H_2O$ were prepared daily.

The RNA was diluted to 100 μg/ml in glass-distilled $H_2O$ and kept at 0°. Amounts of iron ranging from 6400 to 3 moles per mole TMV-RNA (or from about 1 to 0.0005 mole per mole nucleotide) were added to 1 ml of the diluted RNA. The reaction mixtures were kept in an ice bath either in the dark or under the light from a Hanovia fluoro lamp (31300, 125 w) placed 10 cm from the solution (behind 0.5 cm of a 2% $CuSO_4$ solution serving as a filter for wavelengths below 3500 A). Aliquots were taken at various times and assayed for infectivity (Fraenkel-Conrat and Singer, 1959). They were applied directly to test plants after suitable dilution with either 0.1 M (*p*H 7) sodium phosphate buffer, or with the same buffer containing EDTA to complex the metal. More frequently the aliquots were reconstituted before assay (Fraenkel-Conrat and Singer, 1959).

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[1] Abbreviation used in this work: TMV, tobacco mosaic virus.