Photoreactions of Pseudouridine 3′-Phosphate†

LaDonne H. Schulman,‡ Ira Kućan,§ Bennett Edelman, and Robert W. Chambers*

ABSTRACT: At 254 nm, pseudouridine 3′-phosphate undergoes two photochemical reactions. The first involves photolysis to give inorganic phosphate, 5-formyluracil, and 4-hydroxycrotonaldehyde. The second reaction involves rearrangement to a phosphate-containing photoproduct, Xp, whose structure is still unknown, but whose properties are very simlar to that of the starting material. Detailed analysis of kinetic data indicates that Xp is *not* a mandatory intermediate for the release of inorganic phosphate. The cleavage reaction and the rearrangement occur as simultaneous, unimolecular photoreactions. The reaction cross sections (σ), the quantum yields (Φ or ϕ), and the partition coefficient (α) have been evaluated for these photoreactions occurring at 254 nm in dilute, aqueous solution, pH 5.6, at room temperature. Their values are: total pseudouridine 3′-phosphate photochemistry, $\sigma_1 + \sigma_2 = 0.250 \pm 0.005$ cm$^2/\mu$E, $\Phi = 0.0162$; cleavage reaction, $\sigma_1 = 0.16$ cm$^2/\mu$E, $\phi_1 = 0.01$; rearrangement, $\sigma_2 = 0.09$ cm$^2/\mu$E, $\phi_2 = 0.006$; $\alpha = \sigma_1/(\sigma_1 + \sigma_2) = 0.64$.

Pseudouridine 3′-phosphate (I) undergoes photolysis at 254 nm to give 5-formyluracil (II), inorganic phosphate, and a fragment (Y) of unknown structure derived from the remainder of the ribose moiety (Tomasz and Chambers, 1964) (Scheme I). This reaction is of interest from a purely

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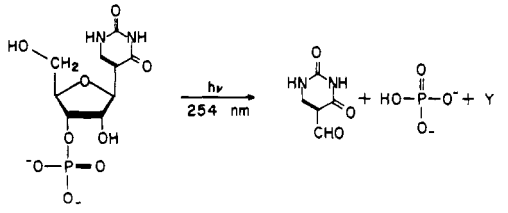


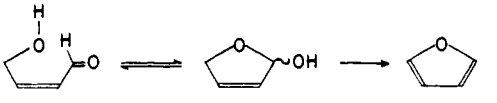
chemical point of view since it represents a marked departure from the photochemistry usually observed with pyrimidines (hydration and dimerization), and it provides an interesting example of bond cleavage occurring in a part of the molecule quite far removed from the absorbing chromophore. The reaction is also of biochemical interest since it leads to cleavage of tRNA at its pseudouridylate residues (Tomasz and Chambers, 1966), and this may be one of the reactions leading to loss of aminoacyl acceptor activity when tRNA is irradiated with ultraviolet light (Scott and Turter, 1962).

In order to provide a quantitative background for examining this reaction in tRNA, we have studied the photolysis of 3′-ΨMP in considerable detail. In this paper, we will provide evidence that the fragment, Y, derived from the ribose moiety, is 4-hydroxycrotonaldehyde and that the primary photochemistry of 3′-ΨMP is complex, involving the cleavage reaction shown above and a parallel rearrangement to give a photoproduct with properties very similar to that of starting material. We will also describe a kinetic study of these reactions in which the reaction cross sections and quantum yields have been evaluated in order to provide a quantitative baseline for projected studies on the photochemistry of individual pseudouridylate residues in tRNA.

Results

When photolysis of 3′-ΨMP at 254 nm was carried out in $H_2{}^{18}O$, no heavy oxygen was found in the inorganic phosphate released. This shows that the phosphate is formed by C—O cleavage and suggests that an elimination reaction, rather than hydrolysis of an unstable intermediate, is involved in breaking this bond. This, along with some mechanistic guesses, suggested that 4-hydroxycrotonaldehyde, $HOCH_2CH{=}CH{-}CHO$, might be the missing primary product derived from the ribose moiety. This compound, however, is known only in its hemiacetal form (Quennehan and Normant, 1949), and this might, in turn, dehydrate to furan (Scheme II).

SCHEME II



Extraction of the photolysis mixture with spectrograde cyclohexane gave material with an ultraviolet spectrum similar to furan, but the data were not good enough to draw any firm conclusions.

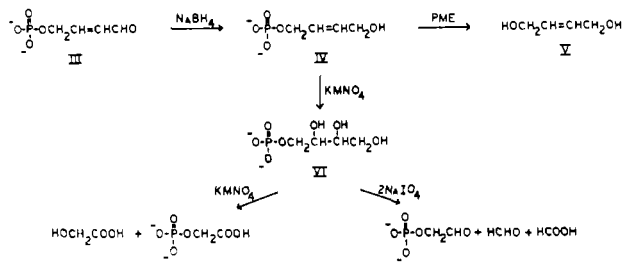
Previous work had shown that photolysis of psuedouridine 3′,5′-dihosphate is very similar to that of 3′-ΨMP and that only the 3′-phosphate group of 3′,5′-ΨMP is lost during irradiation (Tomasz and Chambers, 1964). Thus, the product derived from carbons 2′, 3′, 4′, and 5′ of the ribose moiety should be released with a phosphate group attached to it. This should make the detection of this fragment relatively easy. Furthermore, the presence of a phosphate group in

† From the Department of Biochemistry, New York University School of Medicine, New York, New York 10016. *Received June 8, 1972.* This work was supported by grants from the U. S. Public Health Service (3 R01 GM07262-12), the Health Research Council of the City of New York (U-1530), and the American Cancer Society (NP-32C).

‡ Present address: Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, N. Y. This work was carried out during the tenure of a U. S. Public Health Service postdoctoral fellowship (GM-25,161).

§ Present address: Institute "Rudjer Bošković," Zagreb, Yugoslavia.

CHART I



the 4 position should prevent cyclization to the hemiacetal. Therefore, the diphosphate seemed a more suitable substrate than 3′-ΨMP for identifying the fragment.

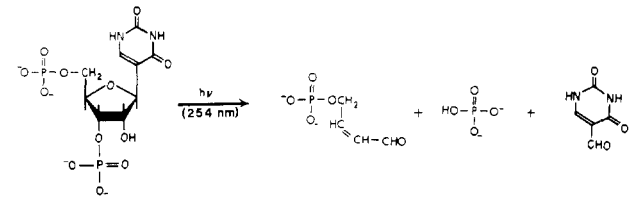
After irradiation of 3′,5′-ΨDP at 254 nm, the pyrimidine derivatives (material and photoproducts) were removed by adsorption on charcoal. The remaining solution was examined spectrophotometrically, and a band with a λ_{max} at 223 nm was found (authentic crotonaldehhde, λ_{max} 223 nm). However, a large number of compounds absorb in this region, so the spectral data were not convincing evidence for an α,β-unsaturated aldehyde. More rigorous characterization was provided by the reaction shown in Chart I.

Fractionation of the photolysis mixture by paper chromatography revealed a compound, III, which gave a positive test for organic phosphate and for an aldehyde (see Experimental Section). The aldehyde function was reduced with $NaBH_4$, and the photoproduct, IV, was treated with phosphomonoesterase. The resulting compound, V, had the same mobility as authentic but-2-ene-1,4-diol on paper chromatograms. Controlled oxidation of IV by $KMnO_4$ gave a new compound, VI, which took up 2 mol of IO_4^-/mol of phosphate and was converted to glycolaldehyde phosphate, formaldehyde, and formic acid. Oxidation of VI with excess $KMnO_4$ have glycolic acid phosphate and glycolic acid. On the basis of this evidence, photoproduct III was assigned the structure of crotonaldehyde 4-phosphate.

Like the other photoproducts in this reaction, the yield of crotonaldehyde 4-phosphate is considerably less than the theoretical amount. In fact, only about 20% of the ribose moiety was accounted for by the yield we obtained in these experiments. Reasons for this are not clear. Model studies with crotonaldehyde indicate that crotonaldehyde 4-phosphate probably undergoes further photochemistry. However, it has not been possible to prepare enough crotonaldehyde 4-phosphate to study the kinetics of this reaction in detail, and it is not clear whether secondary photoreactions are entirely responsible for the low yield or whether other primary photoproducts are formed from the ribose residues.

Identification of crotonaldehyde 4-phosphate as a photoproduct, along with our previous work, enables us to account for all of the atoms in the 3′,5′-ΨDP as shown in Scheme III.

SCHEME III



In order to provide a quantitative basis for our projected studies on the photochemistry of pseudouridine residues in tRNA, we have studied the kinetics of this reaction using the more readily available substrate, 3′-ΨMP. The expected unimolecular photoreaction is given by the scheme A.

$$\Psi p \xrightarrow{\sigma} P_i + \text{other products} \qquad (A)$$

The relevant rate equations are

$$-d[\Psi p]/d\bar{L} = d[P_i]/d\bar{L} = -\sigma[\Psi p] \qquad (1)$$

$$\log ([\Psi p]/[\Psi p]_0) = -\sigma\bar{L}/2.303 \qquad (2)$$

$$\log [1 - ([P_i]/[\Psi p]_0)] = -\sigma\bar{L}/2.303 \qquad (3)$$

where σ = reaction cross section, $\bar{L}$ = average exposure, and $[\Psi p]_0$ = molar concentration of 3′-ΨMP at $\bar{L} = 0$. Thus, both the disappearance of starting material and the formation of P_i should give first-order kinetics. However, when the disappearance of 3′-ΨMP was measured, a semilog plot of the data (Figure 1) did not give a linear curve as required by eq 2. Nor did the release of P_i shown in Figure 2 show first-order kinetics as required by eq 3. These results rule out reaction A and indicate the presence of a photoproduct, Xp, that is not separating from the starting material with the analytical systems (paper chromatography, paper electrophoresis, and ion exchange). The data show that Xp is a uv-absorbing, phosphate-containing photoproduct with properties very much like starting material. This suggests that photoisomerization might have occurred.

In order to see if any of the known isomers of pseudouridine (Cohn, 1960; Chambers, 1966)[1] were formed, the material behaving like starting material after irradiation was isolated by paper chromatography and dephosphorylated with phosphomonoesterase. The nucleosides were fractionated under conditions known to separate the isomers of pseudouridine (Cohn, 1960). As shown in Figure 3, a product resembling α-pseudouridine A was found. However, isolation of this material and rechromatography under slightly different conditions showed clearly that photoproduct X did not correspond to any of the known isomers (Figure 4).

We have not yet isolated sufficient quantities of this photoproduct to determine its structure, but some of its properties are interesting. Its uv spectrum resembles that of α-pseudouridine A (Cohn, 1960). It contains a phosphate group. The nucleoside derived from it by enzymatic dephosphorylation is also formed by irradiation of pseudouridine itself at 254 nm. Like pseudouridine, it isomerizes in hot acid to give products that resemble the pseudouridine isomers (Cohn, 1960). From this information, it appears that Xp is a photorearrangement product of 3′-ΨMP.

There are two, and only two, *simple* schemes that will accommodate Xp as a rearrangement product of 3′-ΨMP. Either Xp is a mandatory intermediate in the release of P_i (scheme B) or it is not (scheme C). Scheme B represents two

[1] These isomers are 5-α-D-ribopyranosyluracil = α-pseudouridine A; 5-α-D-ribopyranosyluracil = β-pseudouridine A; 5-α-D-ribofuranosyluracil = α-pseudouridine; 5-β-D-ribofuranosyluracil = pseudouridine (naturally occurring isomer). Cohn (1960) originally designated these isomers A_F, A_S, B, and C in order of their elution from an ion-exchange column. Our starting material was shown to be pure 5-β-D-ribofuranosyluracil.

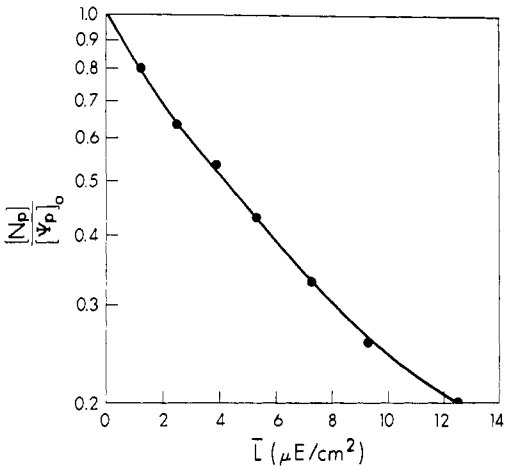


FIGURE 1: Disappearance of pseudouridine 3′-phosphate during irradiation in water, pH 5.6; semilog plot. The irradiation was carried out as described in the Experimental Section. An aliquot containing approximately 0.1 A_{260} unit was removed after each exposure and analyzed for the remaining nucleotide with a nucleotide analyzer, as described in the legend to Figure 5. The nucleotide fraction (Np), which proved to be a mixture of 3′-ΨMP and Xp, was eluted with one column volume of eluent (see Figure 5a for a typical analysis). Calculation of [Np] was based on a pseudouridine standard. $[\Psi p]_0$ = initial concentration of 3′-ΨMP; $\bar{L}$ = average exposure, as defined in the Experimental Section.

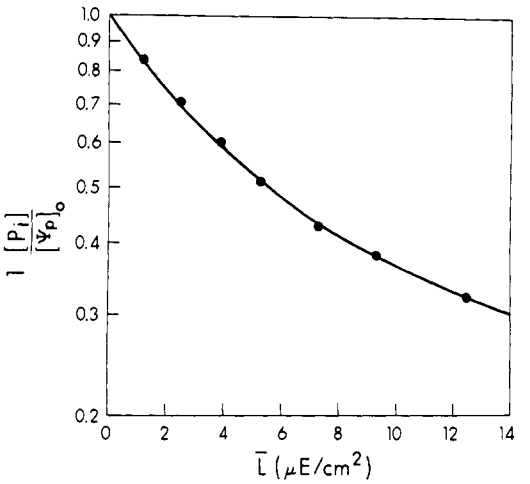


FIGURE 2: Release of inorganic phosphate from pseudouridine 3′-phosphate under the same conditions as in Figure 1 (semilog plot). The details are described in the Experimental Section.

$$\Psi p \xrightarrow{\sigma_1} Xp \xrightarrow{\sigma_2} P_i + \text{other products} \qquad (B)$$

$$\Psi p \xrightarrow{\sigma_1} P_i + \text{other products}$$
$$\Psi p \xrightarrow{\sigma_2} Xp \xrightarrow{\sigma_3} P_i + \text{other products} \qquad (C)$$

consecutive, unimolecular reactions, leading finally to P_i. The kinetics for this type of scheme are well known (Frost and Pearson, 1961). The scheme predicts a lag in the formation of P_i. Our data show no such lag, and we conclude that the photochemistry of 3′-ΨMP does not follow scheme B.[2]

Scheme C represents a more complex situation. The pertinent rate equations are

$$\frac{-d[\Psi p]}{d\bar{L}} = (\sigma_1 + \sigma_2)[\Psi p] \qquad (4)$$

$$d[Xp] = \sigma_2[\Psi p] \qquad (5)$$

$$\frac{-d[Xp]}{d\bar{L}} = \sigma_3[Xp] \qquad (6)$$

$$\frac{-d[P_i]}{d\bar{L}} = \sigma_1[\Psi p] + \sigma_3[Xp] \qquad (7)$$

It can be shown that

$$1 - \frac{[P_i]}{[\Psi p]_0} = re^{-(\sigma_1+\sigma_2)\bar{L}} + (1 - r)e^{-\sigma_3\bar{L}} \qquad (8)$$

where

$$r = \frac{\sigma_1 - \sigma_3}{\sigma_1 + \sigma_2 - \sigma_3}$$

$$1 - r = \frac{\sigma_2}{\sigma_1 + \sigma_2 - \sigma_3}$$

In order to demonstrate that the data fit eq 8, as well as to determine the relative rates of the various components of this complex kinetic system, the reaction cross sections,

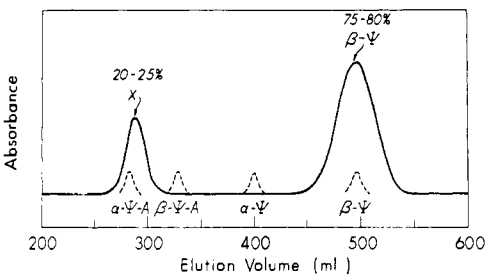


FIGURE 3: Isolation of an unknown photoproduct, X, after dephosphorylation of the nucleotide fraction isolated after irradiation of pseudouridine 3′-phosphate with 254 nm of light. Ultraviolet-absorbing material (21 A_{260} units) behaving like starting material was recovered by paper chromatography in solvent A (see Experimental Section) after irradiation to 63% of theory based on release of P_i. After dephosphorylation with *E. coli* phosphomonoesterase (3.6 μmol of P_i released), the products were chromatographed on a 1 × 5 cm column of Dowex 1-X8 (Cl^-) (200–400 mesh). Elution was carried out with a linear gradient generated with 300 ml of 0.005 M $K_2B_4O_7$–0.02 M NH_4OH in the mixer and 300 ml of 0.02 M NH_4Cl in the reservoir. The flow rate was 1 ml/min. The effluent was monitored with a GME absorption meter (Gilson Medical Electronics). The absorbance is in arbitrary units. The dotted peaks indicate the approximate positions of authentic markers of known pseudouridine isomers.

[2] This statement by be verified by evaluating the equation

$$1 - \frac{[P_i]}{[\Psi p]_0} = \frac{\sigma_1}{\sigma_1 - \sigma_2}e^{-\sigma_2\bar{L}} - \frac{\sigma_1}{\sigma_1 - \sigma_2}e^{-\sigma_1\bar{L}}$$

for σ_1 = 0.25 cm²/μE (determined directly from the disappearance of 3′-ΨMP as described in the text) and various assumed values for σ_2. Even for $\sigma_2 \gg \sigma_1$, where the lag in production of P_i is not pronounced, the shape of the curve is very different from the experimental data given in Figure 2.

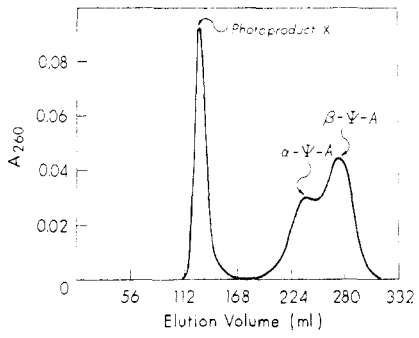


FIGURE 4: Rechromatography of photoproduct X in the presence of authentic samples of α- and β-pseudouridine A (Cohn's A_E and A_S, 1960).[1] The column was 0.6 × 20 cm Dowex 1-X8 (Cl^-) (200-400 mesh) eluted with a linear gradient generated from 300 ml of 0.005 M $K_2B_4O_7$–0.02 M NH_4OH–0.01 M NH_4Cl in the mixer and 0.00375 M $K_2B_4O_7$–0.015 M NH_4OH–0.0125 M NH_4Cl in the reservoir. The effluent was monitored at 260 nm with a Beckman DB spectrophotometer and a Honeywell Elektronic 16 ARU recorder set for a full scale of 20 mV (0.1 *A* unit). α-Ψ-A = 5-α-D-ribopyranosyluracil; β-Ψ-A = 5-β-D-ribopyranosyluracil.

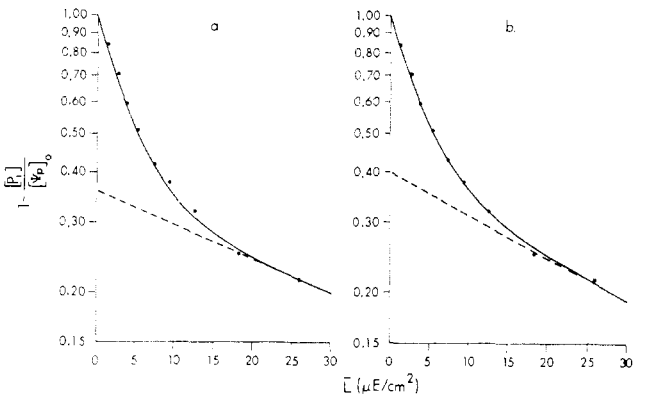


FIGURE 6: Analysis of the complex kinetics for release of inorganic phosphate from pseudouridine 3′-phosphate. (a) The theoretical curve (solid line) obtained for $\sigma_1 + \sigma_2 = 0.250$ cm²/μE, $\sigma_3 = 0.020$ cm²/μE, and $r = 0.64$. The dashed line was used to estimate σ_3 according to eq 9. (b) The theoretical curve (solid line) for $\sigma_1 + \sigma_2 = 0.250$ cm²/μE, $\sigma_3 = 0.025$ cm²/μE, and $r = 0.60$. The dashed line represents eq 9 for these values of σ_3 and r.

σ_1, σ_2, and σ_3, need to be evaluated. This cannot be done directly because cleavage (σ_1) and rearrangement (σ_2) are parallel reactions, and only $\sigma_1 + \sigma_2$ can be obtained by measuring the disappearance of 3′-ΨMP. Furthermore, Xp has not been isolated free of 3′-ΨMP, so σ_3 cannot be evaluated directly. In spite of these difficulties, it is possible to obtain reasonable estimates of these constants by the following kinetic analysis.

First, $\sigma_1 + \sigma_2$ was evaluated. In order to do this, it is necessary to measure the disappearance of 3′-ΨMP (eq 4). However, it is not possible to simply measure the remaining 3′-ΨMP since the presence of the photoproduct, Xp, which we are unable to separate from the starting material, interferes with the determination. Therefore, the necessary analysis was performed on the *nucleosides* produced by dephosphorylation of the nucleotides in the reaction mixture, using an ultramicro ion-exchange system similar to that described by Uziel *et al.* (1968). A typical result is shown in Figure 5. Figure 5a shows the position of the *nucleotides*, as well as the primary photoproduct, 5-formyluracil, and the secondary photoproducts, 5-hydroxymethyluracil, compound W (structure unknown), and uracil-5-carboxylic acid (Tomasz and Chambers, 1966). Figure 5b shows the position of the nucleosides produced by dephosphorylation of the nucleotides. Ψ and X are sufficiently well resolved to give good quantitative data for Ψ. It should be noted by comparing Figure 5a,b that uracil-5-carboxylic acid is the only photoproduct running in the *nucleoside* position, and its concentration is negligible at the exposures used in these experiments.

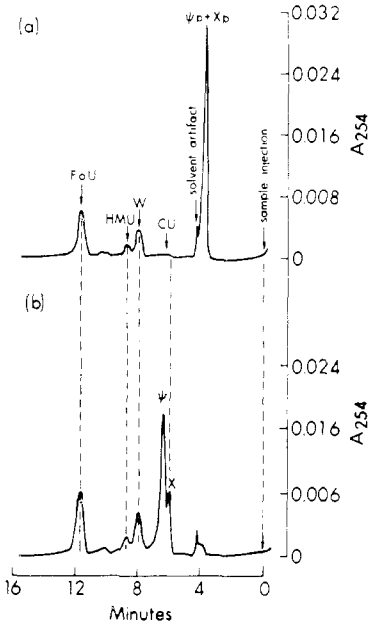


FIGURE 5: Ultramicro ion-exchange analysis of pseudouridine 3′-phosphate and its photoproducts. (a) Direct analysis of the reaction mixture. (b) Analysis after dephosphorylation with alkaline phosphatase. The nucleoside analyzer was modified from that described previously (Kučan *et al.*, 1971) using a fixed-wavelength (254 nm) photometer (Chromatronix, Inc.) with microbore Teflon tubing to monitor the column. The nanomoles of material are proportional to peak height. FoU = 5-formyluracil, HMU = 5-hydroxymethyluracil, CU = uracil-5-carboxylic acid, and X and W = unknown photoproducts. Ψ = pseudouridine.

Using this rapid and sensitive analysis for Ψ, first-order kinetics for the disappearance of 3′-ΨMP were obtained, as required by eq 4. The reaction cross section, $\sigma_1 + \sigma_2$, was evaluated from the integrated form of eq 4 using a semilog plot, fitted by least squares to seven experimental points, covering exposures up to 9 μE/cm²; no point deviated by more than 2%, and the standard error of estimate was 7.6×10^{-3}. These data gave $\sigma_1 + \sigma_2 = 0.250 \pm 0.005$ cm²/μE (95% confidence level).

Next, we evaluated σ_3 and r as follows. Equation 8 indicates that if $\sigma_1 + \sigma_2 \gg \sigma_3$, as our data clearly indicate, then at high exposures, where essentially all of the starting material has reacted, eq 8 simplifies to eq 9, written as its linear transform

$$\log [1 - ([P_i]/[\Psi p]_0)] \approx -(\sigma_3 \bar{L}/2.303) + \log (1 - r) \quad (9)$$

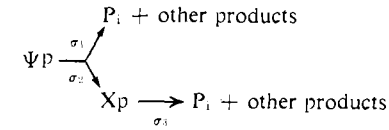
To utilize this approximation, we extended our measurements to higher exposures, and using these data, σ_3 and $1 - r$ were evaluated from the last two points shown in Figure 6a (dashed line); $\sigma_3 = 0.020$ cm²/μE, $1 - r = 0.36$, $r = 0.64$.

With these parameters, it is possible to test our kinetic data for the release of P_i to see if it fits scheme C. Equation 8 was used to generate the theoretical curve for $\sigma_1 + \sigma_2 = 0.25$ cm²/μE, $\sigma_3 = 0.020$ cm²/μE, $r = 0.66$. A semilog plot

of this curve is shown as the solid line in Figure 6a. The fit is fair. Since there is some uncertainty in evaluating $1 - r$ and σ_3 from the two points shown in Figure 6a, we searched for a better fit to our data by *small* adjustments in the line representing eq 9. We found the values, $\sigma_3 = 0.025$ cm²/μE, $1 - r = 0.40$ (giving the dashed line shown in Figure 6b), used with $\sigma_1 + \sigma_2 = 0.250$ cm²/μE (determined directly), gave an excellent fit to our experimental data, as shown by the solid line in Figure 6b. Thus, our kinetic data are consistent with scheme C, but not Scheme B.

The reaction cross sections evaluted in this manner are summarized in Table I. The quantum yields were calculated from the appropriate reaction cross sections and the molar extinction coefficient of 3′-ΨMP at 254 nm (6750 l. mol^{-1} cm^{-1}, in water), according to the equation, $\Phi = \sigma/(2.303) \times (\epsilon_{254} \times 10^{-3})$.[3] The values obtained are also summarized in Table I. It should be noted that the quantum yield for conversion of Xp to inorganic phosphate and other photoproducts cannot be calculated at this time since ϵ_{254} for Xp is unknown.

TABLE I: Reaction Cross Sections and Quantum Yields[a] for the Photoreactions of Pseudouridine 3′-Phosphate at 254 nm according to



$\sigma_1 + \sigma_2 = 0.250 \pm 0.005$ cm²/μE	$\Phi = 1.62 \times 10^{-2}$
$\sigma_1 = 0.16/\mu E$	$\phi_1 = 1 \times 10^{-2}$
$\sigma_2 = 0.09/\mu E$	$\phi_2 = 6 \times 10^{-3}$
$\sigma_3 = 0.025/\mu E$	ϕ_3 = not determined

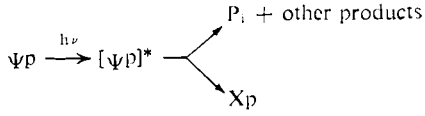
$$\alpha = \sigma_1/(\sigma_1 + \sigma_2) = 0.64$$

[a] These constants are valid only for a dilute (~10^{-4} M), aqueous solution at 25–30° and a starting pH 5.6. Because σ_3 has not been measured directly and because of its small value, there is considerable uncertainty in this particular value. This will have very little effect on the values calculated for σ_1 and σ_2.

Discussion

The data show that the photolysis of 3′-ΨMP is more complex than we originally thought (Tomasz and Chambers, 1964; 1966). Identification of a previously unrecognized primary photoproduct, Xp, that accumulates during the irradiation represents an important finding. The kinetic data for the release of P_i show that Xp is not a mandatory intermediate in the production of P_i. From our current information, the primary photochemistry of 3′-ΨMP can be described by two major photoreactions (Scheme IV).

It seems likely that we are dealing with a partitioning of the excited state between two reaction paths



Our data do not prove this, of course, and we cannot rule out alternatives such as a reaction occurring from an upper vibrational level, rather than first excited state. It is interesting, however, that these same products are produced by acetone-sensitized photochemistry at 312 nm (Kućan and Chambers, 1972, and unpublished results). This shows that the partitioning *can* occur from the triplet state, but it does not prove that it *must* occur from this state.

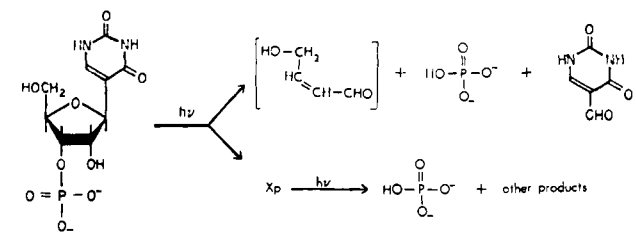
The partition coefficient, α, can be calculated from the appropriate reaction cross sections, $\alpha = \sigma_1/(\sigma_1 + \sigma_2)$. From the data shown in Table I, $\alpha = 0.64$. Thus, the rearrangement represents almost 1/3 of the initial photoreaction under the conditions used (254 nm of H_2O, pH 5.6). Thus, the formation of Xp will have to be considered in studying the photochemistry of Ψ residues in tRNA.

We emphasize that the measurements reported here were made in dilute (~10^{-4} M), unbuffered, aqueous solution at pH 5.6. Appreciable pH changes (several tenths of a pH unit) usually accompany these irradiations. This change may be important, for we have found that the photolysis reaction is pH dependent. Our study of this effect is not yet complete but it clearly involves the 2° phosphate ionization only. This effect does not alter our conclusions, but it does mean that the *values* of the constants summarized in Table I should be regarded with caution; they apply *only* to the conditions specified.

In addition, there is some uncertainty in the values for several of the reaction cross sections and the quantum yields calculated from them. The value for $\sigma_1 + \sigma_2$ and the quantum yield, ϕ, calculated from it, should be quite accurate since the reaction cross section was evaluated directly by measuring the first-order kinetics for the disappearance of 3′-ΨMP. The value for σ_3, on the other hand, could not be obtained by direct measurements because Xp has not yet been isolated free of starting material. Therefore, it was necessary to estimate its value by curve fitting. The data leave some uncertainty in the value, but the small value obtained also means that the contribution of this photoreaction to the total photochemistry is relatively minor, particularly at low exposures. Finally, σ_1 and σ_2, which cannot be measured independently, must be evaluated from the expressions, $\sigma_2 = (\sigma_1 + \sigma_2 - \sigma_3)(1 - r)$ and $\sigma_1 = (\sigma_1 + \sigma_2) - \sigma_2$. Because of the uncertainty in σ_3 and in the quantity, $1 - r$, the values obtained must be regarded as first approximations. In spite of these imperfections in our experiments, the results are sufficiently accurate to provide the necessary quantitative background for studying the photochemistry of individual Ψ residues in tRNA.

Some important pyrimidine photoreactions that may occur in tRNA are summarized in Table II. It is clear from the data that pseudouridine is potentially important on a per residue

SCHEME IV



[3] Φ refers to the total photochemistry of 3′-ΨMP, ϕ refers to individual reactions.

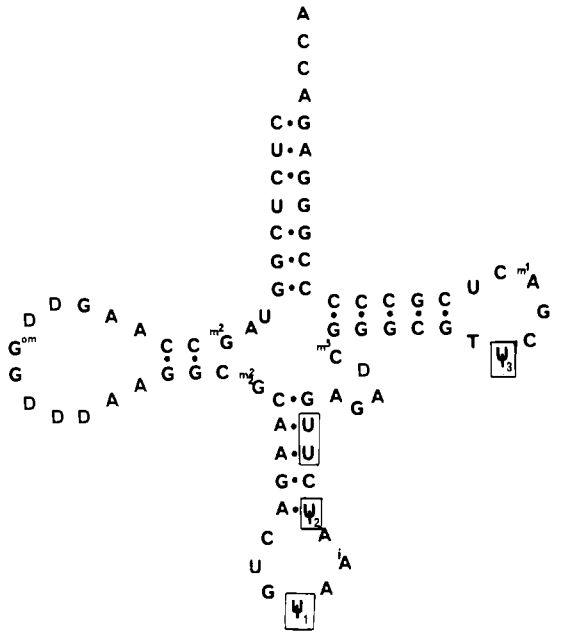


FIGURE 7: Structure of yeast $tRNA^{Tyr}$ (Madison *et al.*, 1966) showing potential UU and Ψ phototargets.

TABLE II: A Comparison of Some Important Pyrimidine Photoreactions.

	$\Phi \times 10^3$		
Compound	Hydrate	Dimer	Total
Up	18[a]		18[a]
Cp	12[b]		12[b]
UpU	18[c]	38[d]	56
CpCp	*e*	*e*	7.5[f]
CpUp	*e*	*e*	18[f,g]
	Cleavage	Rearrangement	
3′-ΨMP	10	6	16

[a] Wierzchowski and Shugar (1959). [b] Becker *et al.* (1967). [c] Brown *et al.* (1966). Calculated from $\sigma_{254} = 0.375$ cm²/μE and $\epsilon_{254} = 9020$ for UMP. [d] Calculated from the sum of the σ_{254} values for the three dimers that were detected (see ref c) using $\epsilon_{254} = 9020$. [e] No data available at 254 nm. [f] These data are collected in McLaren and Shugar (1964), Chapter VII. They are compiled from measurements of absorption changes. [g] Sum of Φ (C residue) = 8 and Φ (U residue) = 10.

basis in tRNA photochemistry. Of the major targets expected in tRNA, only UpU exceeds the reactivity of pseudouridine significantly. We can expect pseudouridine residues to be as reactive as CpU for UpC targets, and considerably more reactive than CpC targets. However, the reactivity of a particular target depends not only on its reaction cross section, but also on its "concentration." Thus, in a tRNA containing only one UpU target and three Ψ targets, such as in yeast $tRNA^{Tyr}$ (Figure 7), the *total reactivity* of the Ψ targets is *potentially* equal to that of the UpU target. In addition, ordered structure can alter the reaction cross section of any given residue. For example, we have devised an analytical procedure for determining the reaction cross sections of each Ψ residue in $tRNA^{Tyr}$ shown in Figure 7. Preliminary results indicate that the residue in the anticodon (Ψ_1) undergoes photochemistry, but neither the residue in the anticodon stem (Ψ_2) or in the right-hand loop of the cloverleaf model react at an appreciable rate. It is not known whether the UpU target reacts or not. These data suggest that photochemistry may provide a valuable tool for probing *localized* ordered structure in tRNA. Before the results can be interpreted unambiguously, however, it is necessary to measure the reaction cross sections for Ψ residues in oligonucleotides derived from the tRNA of interest in order to evaluate nearest-neighbor effects. Additional changes in the cross sections for the same residue in tRNA must reflect higher ordered structure effects in the macromolecule. These measurements are nearly completed, and our results will be reported subsequently.

Experimental Section

General Methods. Paper chromatography was carried out on Whatman No. 40 paper using the descending technique. The solvent systems employed were (A) 2-propanol–concentrated NH_4OH–H_2O (7:1:2), (B) isobutyric acid–0.5 M NH_4OH (pH 3.7, 10:6), and (C) 1-butanol–acetic acid–H_2O (4:1:5).

Ultraviolet-absorbing compounds were detected on paper chromatograms with a Mineralite, shortwave ultraviolet lamp. Aldehydes were detected with *o*-dianisidine spray (0.5% solution in glacial acetic acid (Feigel, 1954)). Inorganic phosphate was measured quantitatively as described previously (Reeves *et al.*, 1968). Vicinal hydroxyl groups were detected with periodate–benzidine spray (Viscontini *et al.*, 1955). Phosphate esters were located with a molybdate–perchloric acid spray (Hanes and Isherwood, 1949), followed by irradiation with ultraviolet light (Bandurski and Axelrod, 1951). Paper electrophoresis was carried out on unwashed Whatman No. 3MM paper, as described previously (Tomasz and Chambers, 1966). Ultraviolet spectra were recorded using a Cary 14 spectrophotometer.

Materials. 5-Hydroxymethyluracil and uracil-5-carboxylic acid were obtained as described previously (Tomasz *et al.*, 1965). Pseudouridine 2′:3′-cyclic phosphate and pseudouridine 3′,5′-diphosphate were synthesized as described before (Tomasz and Chambers, 1965) with the following modification. Attempts to remove the formamide from the cyclic phosphate reaction mixture by a molecular sieve (Bio-Gel P2, 200–400 mesh) were unsuccessful. Therefore, adsorption onto water-washed charcoal (196 Barneby-Chaney, Columbus, Ohio) followed by elution with 2-propanol–concentrated NH_4OH–H_2O (50:3:47) was used to separate pseudouridine 2′:3′-cyclic phosphate from formamide, instead of performing electrophoresis directly, as described previously. Pseudouridine 2′:3′-cyclic phosphate is unstable and cannot be stored for extended periods, even at 0°. Therefore, samples were purified just before use by paper chromatography in solvent A.

The isomers of pseudouridine were prepared by ion-exchange chromatography of commerical samples (Calbiochem, Los Angeles, Calif.) (Chambers, 1966). Pseudouridine 2′- and 3′-phosphate were prepared by a modification of Cohn's method (1960; see Chambers, 1966).

Irradiation of Pseudouridine 3′-Phosphate at 254 nm for Kinetic Studies. A solution of pseudouridine 3′-phosphate (approximately 2×10^{-4} M) was irradiated with a low-

pressure, mercury lamp (Spectroline, Model R-51), mounted vertically behind a 2-mm thick Vycor glass filter. The reaction solution was contained in a stoppered quartz cuvet, 1-cm light path, held vertically in a rigid cell holder approximately 3 cm from the surface of the lamp and mixed thoroughly throughout the irradiation by mechanical stirring. The light path through the solution was always 1 cm, regardless of the volume. The average exposure, $\bar{L}$ (the average photon fluence seen by each molecule in the vessel), for each time interval was calculated from the expression, $\bar{L} = \Sigma\Delta\bar{L} = \Sigma(I_0 \cdot F \cdot \Delta t)$ (Johns, 1968), where Δt is the irradiation interval and F is a correction factor for the average light absorbed by the solution during each time interval (Morowitz, 1950). I_0 was determined by uridine actinometry using the quantum yield, 0.021, and the molar extinction coefficient at 254 nm, 9020 l. mol^{-1} cm^{-1}. For the experiments described here, I_0 = 0.34 μE min^{-1} cm^{-2}.

The initial concentration of the pseudouridine 3′-phosphate was determined from its absorption at λ_{max} and its molar extinction coefficient, ϵ_{max} 7900, which, in turn, was determined from its ultraviolet absorption spectrum and total phosphate analysis. After each interval of irradiation, the absorbance was measured by 254 nm with a Cary Model 14 spectrophotometer, and aliquots were removed for determination of inorganic phosphate or nucleoside analysis.

Characterization of Crotonaldehyde 4-Phosphate Produced by Photolysis of Pseudouridine 3′,5′-Diphosphate. Solutions of pseudouridine 3′,5′-diphosphate irradiated in water, as described below, and chromatographed in solvent B yielded a phosphate-containing product, R_F 0.20, not formed from irradiation of Ψ3′P. The product gave a positive test with *o*-dianisidine, indicating the presence of an aldehyde group. It was only partially resolved from unreacted starting material by paper chromatography, but could be readily separated from it by electrophoresis at pH 7, or by charcoal adsorption of 3′,5′-ΨDP. The photoproduct was characterized as crotonaldehyde 4-phosphate, III, by degradation to known compounds, as illustrated in Chart I. The experimental procedure was as follows.

3′,5′-ΨDP (10 ml; 1 A_{260} unit/ml) in a 9-cm diameter petri dish covered with a 2-mm thick Vycor filter and cooled with a fan were exposed to 254-nm light from a low-pressure mercury lamp. The solution was stirred with 0.2 g of charcoal and filtered through a Millipore disk. Chromatography of the filtrate in solvent B yielded only III and P_i. In a separate experiment, the filtrate was reduced to a volume of 4 ml and treated with 5 mg of $NaBH_4$ at room temperature for 3 hr. The reaction was stopped by adjusting to pH 3.5 with Dowex 50W (H^+). The resin was removed by filtration and the boric acid removed by repeated evaporation with methanol. Chromatography in solvent B yielded only P_i and IV, R_F 0.25, which no longer reacted with *o*-dianisidine. The yields of III and IV were improved in subsequent experiments by chromatographing solutions of irradiated 3′,5′-ΨDP in solvent B, eluting all of R_F 0.20 material (contaminated with about half the unreacted starting material) and treating with $NaBH_4$, as described above. Under the conditions used, 3′,5′-ΨDP did not react with $NaBH_4$ and was readily separated from IV by chromatography in solvent B.

When the filtrate obtained from reaction with $NaBH_4$ was treated with *E. coli* phosphomonoesterase prior to chromatography in solvent B, a new compound, V, was obtained, R_F 0.76. The spot was detected with 1% $KMnO_4$ spray and corresponded in mobility to authentic but-2-ene-1,4-diol (Aldrich Chemical Co.).

Compound IV, isolated by paper chromatography in solvent B, was eluted with water and treated with 2 drops of 1% $KMnO_4$ in 10% acetic acid. The pink solution was allowed to stand at room temperature for 90 sec. The reaction was quenched by addition of 1 drop of dilute $Na_2S_2O_3$. Dowex 50W (H^+) was added to remove cations. The solution was filtered and evaporated. Paper chromatography in solvent B yielded VI, R_F 0.15, which contained phosphate and reacted with 5% $NaIO_4$ spray. Compound VI was the only periodate-positive spot detected on chromatograms after oxidation of total irradiation reaction mixtures with dilute $KMnO_4$, as described above. Thus, in subsequent experiments, IV was oxidized without prior isolation. Compound VI was then isolated by chromatography in solvent B. No periodate-positive spots could be detected on paper chromatograms prior to oxidation of IV.

Compound VI was prepared as described above from several combined reaction mixtures of irradiated 3′,5′-ΨDP. The product was isolated by paper chromatography in solvent A, eluted with water, and analyzed for phosphate. The sample was then oxidized with $NaIO_4$, using the spectrophotometric method of Dixon and Lipkin (1954). Trial experiments with glycerol and α-glycerol phosphate gave values of 1.8 mol of IO_4^- consumed/mol of glycerol and 0.9 mol of IO_4^-/mol of α-glycerol phosphate. Oxidation of the solution of VI yielded a value of 2.1 mol of IO_4^- consumed/mol of phosphate.

Further oxidation of VI with $KMnO_4$ gave two additional products which were characterized as follows. One product contained phosphate, gave a color reaction with 0.3% Bromophenol Blue in 0.01 M citric acid spray, and showed the same mobility on electrophoresis at pH 7.1 in 0.05 M phthalate buffer as phosphoglycolic acid prepared by the method of Fleury and Courtois (1941). The other $KMnO_4$ oxidation product showed a weak reaction with the $NaIO_4$ spray and the acid indicator spray. Its R_F in solvent A (0.47) and in solvent C (0.63) was the same as that of glycolic acid. It was further characterized as glycolic acid by its reaction with chromotropic acid using the method of Fleury *et al.* (1953).

Irradiation of Ψ3′P in $H_2{}^{18}O$. Ψ3′P was added to $H_2{}^{18}O$ (Bio-Rad, 1.636 atom % excess ^{18}O) to give a solution containing 1.5 A_{260}/ml. An aliquot of this solution (133 ml) was added to a water-jacketed reaction chamber and stirred by bubbling prepurified nitrogen gas through the solution. A low-pressure mercury lamp, immersed in 20% acetic acid separated from the sample solution by a quartz sleeve, was used as a source of 254 nm of light. The sample was irradiated for 30, 60, and 90 min, yielded 57, 75, and 84% release of inorganic phosphate, respectively. After 90-min irradiation, the $H_2{}^{18}O$ was recovered by vacuum distillation. The residue was dissolved in 2.5 ml of water, the inorganic phosphate precipitated as $MgNH_4PO_4$, and the filtrate drawn off. The $MgNH_4PO_4$ was freed of Mg^{2+} with Dowex 50W (H^+) resin and the sample was neutralized to pH 4.4–4.6 with KOH. The KH_2PO_4 was precipitated by addition of alcohol (Boyer *et al.*, 1955), and the ^{18}O content of a dried sample was determined by mass spectrometry as described by Boyer *et al.* (1961). The inorganic phosphate released on irradiation of Ψ3′P under these conditions was found to contain 0.025% excess ^{18}O, indicating incorporation of 6.1% ^{18}O into the sample. In a control experiment, a solution of H_3PO_4 containing 2.2 μmol of P_i/ml was irradiated for 90 min and reisolated as described above. The recovered inorganic phosphate was found to contain 0.006% excess ^{18}O, indicating incorporation of 1.5% ^{18}O into the sample.

Acknowledgments

We are indebted to Dr. Peter Hinkel for the mass spectra analysis and to Drs. R. C. Warner and G. B. Weiss for assistance with the mathematics and computer programming.

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Use of Phosphate-Blocking Groups in Ligase Joining of Oligodeoxyribonucleotides†

C. L. Harvey, R. Wright, A. F. Cook, D. T. Maichuk, and A. L. Nussbaum*

ABSTRACT: The polynucleotide ligase from bacteriophage T_4 is able to join oligomers in which those terminal phosphate groups not directly involved in the formation of the new phosphodiester bond are in the form of alkyl phosphorothioates. These latter may be helpful in preventing alternate wrong joinings and serving as a handle for subsequent fragment modification.

One current strategy for the construction of deoxyribonucleotide duplexes ("genes") large enough and of the proper primary sequence to contain information that can, in principle, be transcribed into biologically nontrivial RNA involves the chemical synthesis of oligomers of sufficient size to be joined into larger arrays enzymatically (Agarwal *et al.*, 1970.)[1] This operation, catalyzed by polynucleotide ligase, requires that two segments to be joined must be held in adjacent positions by separately associating, *via* conventional antiparallel Watson–Crick bonding, with a third fragment (the "splint") of appropriate complementary sequence so that the 3′-hydroxyl group of one (the "acceptor") is brought into close juxtaposition to the 5′-terminal phosphate of the other (the "donor"). The splint thus provides specific template guidance for the ligation proper.

There have been a few observations that *in vitro* joining may deviate from this scheme. Thus, it was found (Tsiapalis and Narang, 1970) that the fidelity of the joining is not perfect; the ultimate base on the oligomer acceptor does not have to be complementary to the corresponding counterbase on the splint. Furthermore, certain types of duplex "end-to-end" joining or terminal cross-linking were found to be complicating

† From the Chemical Research Department, Hoffmann-La Roche Inc., Nutley, New Jersey 07110. *Received July 13, 1972.* This publication constitutes paper IX in a series entitled Nucleoside Phosphorothioates. For paper VIII, see Heimer *et al.*, 1972a.

[1] Other approaches consist of the isolation of operons by genetic and physicochemical means (Shapiro *et al.*, 1969) or by "reverse" transcription of purified messengers (Ross *et al.*, 1972; Verma *et al.*, 1972; Kacian *et al.*, 1972).