The Chemistry of Pseudouridine. V. Periodate Oxidation of Pseudouridylic Acid and Soluble Ribonucleic Acid*

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ABSTRACT: Both pseudouridine-3′-phosphate and pseudouridine-3′,5′-diphosphate undergo an unusual oxidation by potassium periodate at *p*H 8.9 and 50° to give 5-formyluracil, 5-carboxyuracil, inorganic phosphate, formaldehyde, and unidentified products. As the *p*H is lowered toward 7 the reaction slows down and 5-carboxyuracil becomes the only detectable product absorbing in the ultraviolet above 260 mμ. These results indicate that two different types of periodate oxidation occur under the above conditions.

In addition to these reactions, which are specific for pseudouridine-3′-phosphate and -3′,5′-diphosphate, a slow, nonspecific loss in absorbance (260 mμ) occurs with pseudouridine-3′-phosphate and with uridine-, cytidine-, adenosine-, and guanosine-2′(3′)-phosphates. These nonspecific reactions foiled an attempt to cleave soluble ribonucleic acid at its pseudouridine residues by periodate oxidation.

Pseudouridine,[1] because of its unique structure (I, Chart 1), displays properties which are unusual for a nucleoside. One of the most interesting of these properties is its ability to isomerize in either acid or alkali (Cohn, 1960, 1961; Shapiro and Chambers, 1961). In order to explain this isomerization we have postulated the formation of an open-chain intermediate II which can recyclize in four different ways to give anomeric furanose (I and III) and anomeric pyranose (IV and V) forms.[2]

This hypothesis can be extended to pseudouridine-3′-phosphate (VI, Chart 2) since the 3′-hydroxyl group does not participate directly in the isomerization mechanism. In this case the key intermediate would be the unsaturated derivative VII (Chart 2) which could recyclize in four different ways as before. In support of this, it has been found that pseudouridylic acid (mixed 2′ and 3′) does isomerize in either acid or alkali (Cohn, 1961).

If this theory is correct then it is reasonable to assume that addition of water might compete with the cyclization reaction giving rise to a new open-chain compound, VIII, which contains two α-glycols, a grouping not found in ψ3′P or its isomers. Thus it should be possible to demonstrate the formation of compound VIII, even if it is present at a very low concentration, by conducting a periodate under conditions where isomerization is known to occur.

Preliminary experiments established that no uptake of periodate occurred at low *p*H even at elevated temperatures. At room temperature no oxidation occurred in alkaline solution, but at 50° periodate was consumed.

As shown in Chart 2, the primary products expected from this oxidation are 5-formyluracil (compound IX), tartronic dialdehyde phosphate (compound X), and formaldehyde. However, a phosphate ester α to an aldehyde is often hydrolyzed easily by mild alkali.[3] Therefore, in alkaline solution, tartronic dialdehyde phosphate (compound X) would be expected to hydrolyze to tartronic dialdehyde and inorganic phosphate. The

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[1] The following abbreviations are used in this paper: ψ, pseudouridine (5-β-D-ribofuranosyluracil); ψ3′P, pseudouridine-3′-phosphate; ψ3′,5′DP, pseudouridine-3′,5′-diphosphate; FoU, 5-formyluracil; CU, 5-carboxyuracil; U2′(3′)P, uridine-2′(3′)-monophosphate; C2′(3′)P, cytidine-2′(3′)-monophosphate; G2′(3′)P, guanosine-2′(3′)-monophosphate; A2′(3′)P, adenosine-2′(3′)-monophosphate.

[2] The structure of pseudouridine C (compound I), the naturally occurring isomer, has been established as β-D-ribofuranosyluracil (Yu and Allen, 1959; Cohn, 1959, 1960; Shapiro and Chambers, 1961; Michelson and Cohn, 1962). It follows from its properties that pseudouridine-B is the α anomer (compound III). It has also been established that the A isomers are anomeric 5-D-pyranosyl uracils (Cohn, 1960; Shapiro and Chambers, 1961; Chambers and Kurkov, 1964) and it follows from their properties (Cohn, 1960; Shapiro and Chambers, 1961; Chambers *et al.*, 1963; R. W. Chambers, unpublished data) that pseudouridine A_F probably has the α configuration (compound V) while A_S is β (compound IV).

[3] For example, glycolaldehyde phosphate is hydrolyzed 30% in 1 hour at *p*H 8 and 100°. Glycol phosphate, on the other hand, is stable under these conditions (Fleury and Courtois, 1948).

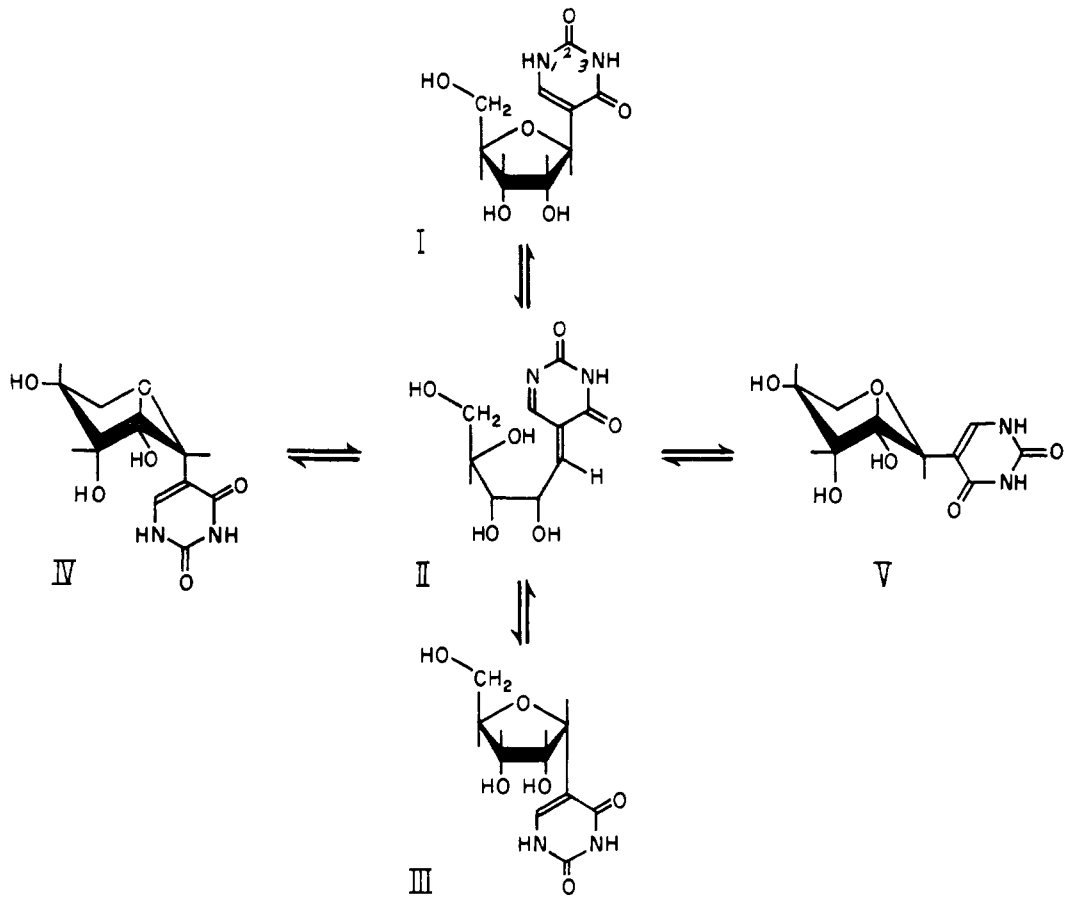


CHART 1: Isomerization of pseudouridine.

dialdehyde would then be oxidized further to formic acid[4]:

$$\mathrm{OHC{-}CH(OPO_3)^{2-}{-}CHO} \xrightarrow{\mathrm{OH^-}} \mathrm{P_i}$$
$$+\ \mathrm{OHC{-}CHOH{-}CHO} \xrightarrow{2\ \mathrm{IO_4^-}} 3\ \mathrm{HCO_2H}$$

According to these predictions, then, periodate oxidation of ψ3'P under alkaline conditions can be formulated as follows:

$$\psi\mathrm{3'P} + 4\ \mathrm{IO_4^-} \longrightarrow \mathrm{FoU} + \mathrm{HCHO} + 3\ \mathrm{HCO_2H} + \mathrm{P_i} + 4\ \mathrm{IO_3^-}$$

This reaction is of interest not only because it provides a critical test of the mechanism we have suggested for isomerization of pseudouridine and pseudouridylic acid, but also because it suggests a method for cleaving s-RNA specifically at its pseudouridine residues. This paper describes experiments designed to test these predictions.

Results and Discussion

In potassium bicarbonate buffer, *p*H 8.9, ψ3'P did not react with potassium periodate at room temperature. At 50°, however, 4.32 eq of periodate were consumed in 23 hours. Analysis of the reaction mixture gave the results shown in Table I.

It can be seen from the data that the predicted products were indeed formed. It is apparent, however, that they were not produced in the quantities expected and that 5-carboxyuracil, which was not predicted, was a major product.

Only about one-third of the expected formaldehyde was detected in the product mixture. It has been reported that formaldehyde is oxidized by periodate under alkaline conditions to formic acid (Hartman, 1954). This reaction did not occur under the conditions we used since heating ethylene glycol with periodate in 0.5 M $KHCO_3$ at 50° for 20 hours gave the theoretical

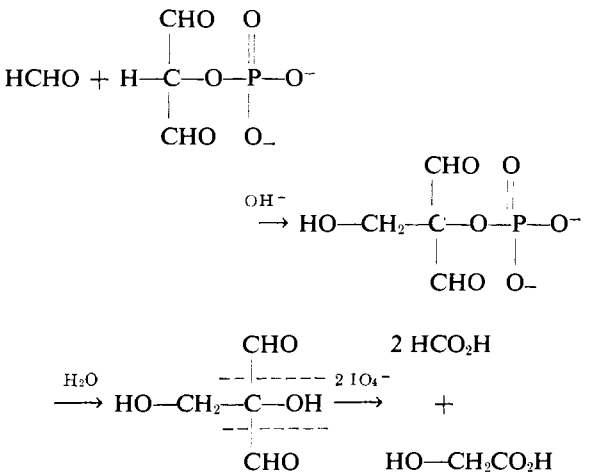
[4] An equally conceivable reaction is the direct hydroxylation of tartronic dialdehyde phosphate:

$$\mathrm{OHC{-}CH(OPO_3)^{2-}{-}CHO} \xrightarrow{\mathrm{IO_4^-}} \mathrm{OHC{-}COH(OPO_3)^{2-}{-}CHO}$$
$$\longrightarrow \mathrm{HPO_4^{2-}} + \mathrm{OHC{-}CO{-}CHO} \xrightarrow{\mathrm{IO_4^-}} \mathrm{HCO_2H} + \mathrm{OHC{-}CO_2H}$$
$$\xrightarrow{\mathrm{IO_4^-}} \mathrm{HCO_2H} + \mathrm{CO_2}$$

For a discussion of this type of reaction see Dyer (1956).

amount of formaldehyde. This also established that the analytical procedure used to detect formaldehyde in the ψ3'P reaction mixture was valid.

It seems most likely that the low yield of formaldehyde was due to aldol-type condensations between the formaldehyde and other fragments from the ribose moiety. One such possible reaction is between formaldehyde and tartronic dialdehyde phosphate[5] followed by hydrolysis of the phosphate ester and periodate oxidation as shown here.



It is also apparent from the data in Table I that more than the expected uptake of periodate occurred and that 5-carboxyuracil was a major product. The most obvious explanation of this "overoxidation" is direct conversion of formyluracil to carboxyuracil. To test this, synthetic 5-formyluracil was treated with potassium periodate under the same conditions used for ψ3'P. After 23 hours less than one-third of the starting material had been oxidized to carboxyuracil (Table II). This rate of oxidation is not fast enough to account for *all* the carboxyuracil found (see Table I).

An alternative pathway for the production of carboxyuracil is a direct oxidative hydroxylation of ψ3'P at carbon 1' to give compound XI (Chart 3).[6] Since there was very little precedent for direct oxidation of this type of carbon,[7] we examined some model compounds under the conditions used for pseudouridylic acid and found that direct hydroxylation is indeed feasible. The results (see Table II and experimental section) established that the following sequence of reactions can occur:

$$\text{thymine} \xrightarrow{IO_4^-} \text{5-hydroxymethyluracil} \xrightarrow{IO_4^-} \text{5-formyluracil} \xrightarrow{IO_4^-} \text{5-carboxyuracil}$$

TABLE I: Periodate Oxidation of Pseudouridine-3'-monophosphate.[a]

	Equivalents	
	Found	Corrected
Periodate uptake	4.32	4.80
Formaldehyde	0.30–0.36	0.33–0.40
5-Formyluracil	0.26–0.30	0.29–0.33
5-Carboxyuracil	0.44	0.5
Inorganic phosphate	0.82–0.83	0.9
Pseudouridine-3'-phosphate recovered	0.09	

[a] Pseudouridine-3'-monophosphate (2.76 μmoles) and KIO_4 (18.0 μmoles) were dissolved in 1.32 ml. of 0.25 M $KHCO_3$ and the solution was heated for 23 hours at 50° in a tightly stoppered test tube. Aliquots from the reaction mixture were analyzed for IO_4^- and P_i as described in the experimental section. A larger aliquot (0.47 ml) was analyzed by ion-exchange chromatography on Dowex 1 (acetate) (0.8 × 3 cm, 200–400 mesh, 8% cross-linked) as follows: Formaldehyde was eluted with water (20 ml), FoU with 0.02 M acetic acid (32 ml), and CU with 1% acetic acid (40 ml); IO_3^- and ψ3'P were eluted together with 0.05 M NH_4Cl (40 ml). The results are expressed in equivalents based on the starting ψ3'P (found) and equivalents corrected for recovered starting material (corrected).

TABLE II: Periodate Oxidation of 5-Formyluracil.[a]

	Equivalents
Periodate uptake	1.16
5-Formyluracil	0.57
5-Carboxyuracil	0.29
Loss of ultraviolet absorption	0.14

[a] 5-Formyluracil (4.3 μmoles) was mixed with 25.5 μmoles KIO_4 in 1.36 ml of 0.2 M $KHCO_3$ and the solution was heated for 23 hours at 50°. The products were determined by ion-exchange chromatography as described in Table I. Equivalents are based on the starting material.

[5] This reaction is analogous to the condensation between benzyloxymalondialdehyde and formaldehyde in sodium bicarbonate solution (Schwartz and McDougall, 1956). The concentrations we used were comparable to those used by Schwartz and McDougall.

[6] It is also possible, though we consider it less likely, that direct oxidation of compounds VII or VIII (Chart 2) occurs. Both of these reactions would lead to compound XIII (Chart 3).

[7] Hydroxylation by periodate of certain compounds containing an active methylene is well known. For a discussion, see Dyer (1956). Similar reactions have been proposed for the overoxidation of α-methyl glucoside (Neumuller and Vasseur, 1953) and of dialdehydes resulting from glycol cleavage of nucleoside 5'-phosphates (D. H. Rammler, personal communication). However, none of these reactions is directly applicable to pseudouridylic acid.

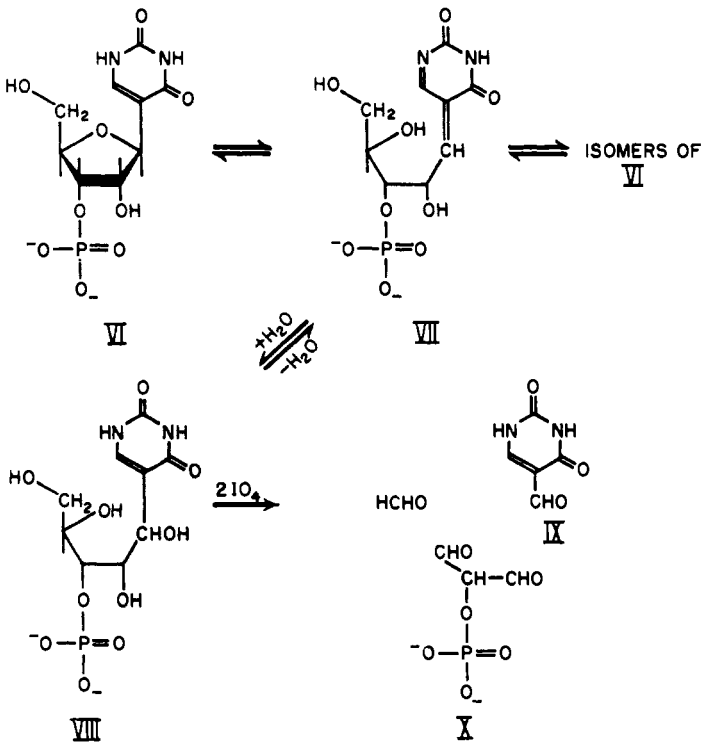


CHART 2: Periodate oxidation of pseudouridine-3′-phosphate.

These results establish the precedent for the first step shown in Chart 3. Of the two possible pathways leading from the hemiketal, XI, to carboxyuracil we favor the one involving the ketone, XIII, as an intermediate. The main reason for this is that esters of carboxyuracil are relatively stable. For example, after 23 hours at 50° in 0.25 M $KHCO_3$, less than 5% hydrolysis of 5-carboxyuracil ethyl ester occurred. Thus if an ester such as compound XII had formed we should have been able to detect it. However, no such intermediate could be found.

Regardless of the actual pathway to carboxyuracil, tartronic dialdehyde phosphate (compound X) can be regarded formally as a product.[8] Thus the reactions leading to formyluracil and to carboxyuracil should produce the same labile phosphate intermediate. The data in Table I indicate that this is the case since more than 90% of the theoretical inorganic phosphate (after correcting for unchanged starting material) was found in the reaction mixture.[9]

It is also apparent by inspection of the reactions shown in Charts 2 and 3 that oxidation and labilization of the 3′-phosphate ester should occur equally well when the 5′-hydroxyl group is esterified since this group does not play any direct role in the reaction. This prediction is borne out by the data in Table III. Comparison of these data with those shown in Table I show that the oxidation of ψ3′,5′DP was similar to that of ψ3′P. It is interesting that the diphosphate was oxidized somewhat more rapidly than the monophosphate.

TABLE III: Periodate Oxidation of Pseudouridine-3′,5′-diphosphate.[a]

	Equivalents
Periodate uptake	4.5
Formaldehyde	0.16
Inorganic phosphate	1.0
5-Formyluracil	0.40
5-Carboxyuracil	0.46

[a] A solution of ψ3′,5′DP (2.1 μmoles) and KIO_4 (13.6 μmoles) in 0.25 M $KHCO_3$ (1.0 ml) was heated for 23 hours at 50°. Analysis was carried out as described in Table I.

Particularly noteworthy is the fact that only 1 eq of P_i was released by oxidation of ψ3′,5′DP. This is as expected for the reactions:

$$\psi 3',5'\text{DP} \xrightarrow{H_2O} \text{FoU} + \xrightarrow{IO_4^-} {}^{2-}O_3POCH_2—CHOH—CH(OPO_3)^{2-}—CHO \xrightarrow{H_2O} {}^{2-}O_3POCH_2—CHOH—CHOH—CHO + P_i$$

erythrose-4-phosphate

[8] Whether this is an *actual* product or not, of course, depends on the relative rates of periodate oxidation and phosphate hydrolysis of intermediates produced at various steps.

[9] No inorganic phosphate was released from ψ3′P in *p*H 8.9 potassium bicarbonate buffer at 50° for 23 hours *in the absence of periodate*, and ψ3′P was the only ultraviolet-absorbing compound that could be recovered from the reaction mixture.

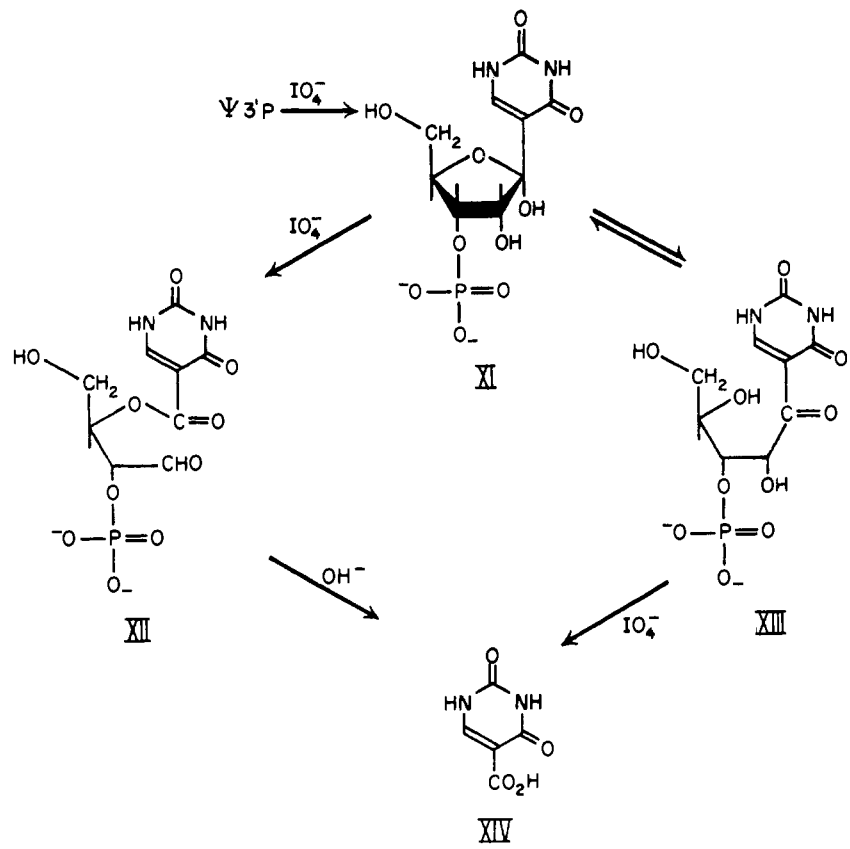


CHART 3: Possible pathways for the formation of 5-carboxyuracil from pseudouridine-3'-phosphate.

However, it should be noted that formaldehyde (0.16 eq) is also formed. This can be explained by further oxidation of the erythrose-4-phosphate to produce a new labile phosphate:

$$^{2-}O_3POCH_2—CHOH—CHOH—CHO \xrightarrow{IO_4^-} {}^{2-}O_3POCH_2—CHO + 2\,HCO_2H$$

$$^{2-}O_3POCH_2—CHO \xrightarrow{H_2O} P_i + HOCH_2—CHO \xrightarrow{IO_4^-} HCHO + HCO_2H$$

This accounts satisfactorily for the production of formaldehyde. However, an additional equivalent of P_i would be produced per equivalent of HCHO. Thus we would have expected 1.16 eq of P_i to be released rather than the 1.0 eq actually found. This discrepancy can be explained by incomplete hydrolysis of erythrose-2,4-diphosphate and glycolaldehyde phosphate.[10]

The successful oxidation of ψ3',5'DP led to the conclusion that it might be possible to cleave s-RNA at its pseudouridine residues as outlined in Chart 4. However, several problems in extending this reaction to s-RNA were already apparent. First, no information on periodate oxidation of ordinary nucleotides under these unusual conditions was available in the literature and nonspecific reactions might interfere with selective cleavage of the pseudouridine residues. Second, it seemed likely that nonspecific alkaline hydrolysis of s-RNA would occur under the conditions used to oxidize ψ3'P (pH 8.9, 50°, 23 hours).[11] Therefore, before attempting to apply periodate oxidation to s-RNA, where it might be difficult to show whether or not specific cleavage occurred, we carried out a series of model experiments designed to test the effect of various reaction variables.

Periodate oxidation of yeast uridylic acid at pH 8.9 in bicarbonate buffer at 50° was examined first. As shown in Table IV, uridylic acid does slowly release about 20% of its phosphate. Furthermore, only about 65% of the nucleotide could be recovered, indicating that degradative reactions were occurring.

Next, the effect of pH on the oxidation reaction was

[10] For example,

$$E\text{-}2,4\text{-}DP \xrightarrow{H_2O} 0.84\,P_i + 0.84\,E\text{-}4\text{-}P \xrightarrow{IO_4^-} 1.68\,HCO_2H + 0.84\,GP$$

$$0.84\,GP \xrightarrow{H_2O} \frac{0.16\,P_i}{1.00\,P_i} + 0.16\,G + \xrightarrow{IO_4^-} 0.16\,HCO_2H + 0.16\,HCHO$$

(E = erythrose; G = glycolaldehyde)

[11] We established that extensive loss of the ability of *E. coli* s-RNA to load phenylalanine occurred at pH 8.9 (bicarbonate) at 50° in the absence of periodate. A slower loss in activity also occurred at pH 7.5. We attribute this to nonspecific hydrolysis.

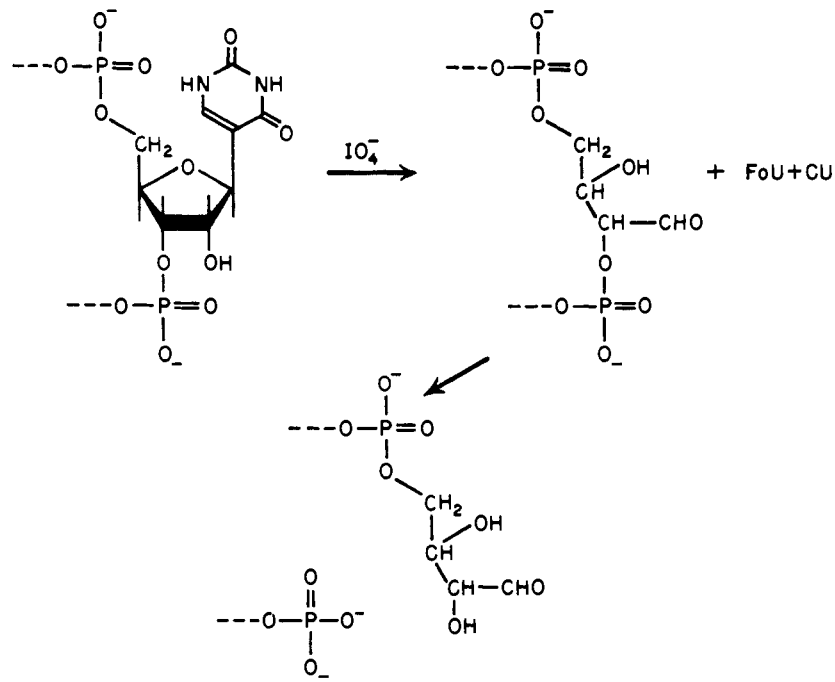


CHART 4: Proposed scheme for cleavage of s-RNA at its pseudouridine residues.

TABLE IV: Periodate Oxidation of Uridine-2′(3′) monophosphate.[a]

	Equivalents
Periodate uptake	0.8–1.6
Inorganic phosphate	0.19–0.21
Recovered U2′(3′)P	0.63–0.69

[a] The reaction and analysis were carried out as described for ψ3′P (see Table I). The only ultraviolet-absorbing product found by ion-exchange chromatography was starting material (eluted with 0.05 M NH_4Cl). No P_i was released and 90% of the starting material was recovered in the absence of KIO_4.

examined. First of all, we found that the rate of oxidation of ψ3′P decreased markedly as the *p*H was lowered. For example, at *p*H 7.5 about 40 hours was required for the reaction to reach completion (Figure 1), compared with about 23 hours at *p*H 8.9 (Table V). Furthermore, the product distribution changed with *p*H. At *p*H 8.9 both formyluracil and carboxyuracil were formed from ψ3′P and about 80% of the starting material was accounted for by ultraviolet-absorbing compounds. At *p*H 7.5 carboxyuracil but not formyluracil was found, and at *p*H 7.0 only a trace of carboxyuracil was found. Furthermore, a large loss of ultraviolet-absorbing materials occurred when the reaction was run at *p*H 7.0–7.5.

The lack of formyluracil formation at *p*H values near neutrality is in agreement with the postulation (Chart 2) that opening of the furanosyl ring is a prerequisite for the production of formyluracil by periodate oxidation. Since the driving force for this ring opening is believed to be removal of a proton from N-1 (Chambers *et al.*, 1963) formation of formyluracil should decrease with *p*H, as was found.

The decrease in rate of carboxyuracil formation with decreasing *p*H is understandable on a similar basis. If oxidative hydroxylation of C-1′ of ψ3′P is the rate-limiting step in carboxyuracil production, then the overall rate will be dependent upon the rupture of the C–H bond at C-1′. This reaction should also be facilitated by formation of the N-1 anion since cleavage of the C–H bond and the C–O bond at C-1′ are formally similar.

Regardless of the actual mechanisms involved, the results in Table V clearly demonstrate that, as the *p*H is lowered, a reaction which destroys the pyrimidine ring becomes increasingly important. Furthermore, this reaction is not confined to ψ3′P. As shown in Figure 1, U2′(3′)P also reacts, although at a slower rate than ψ3′P. It should also be noted (Table V) that the U2′-(3′)P reaction is not affected by lowering the *p*H.

This destruction of the pyrimidine ring presented a serious problem. In order to see what effect the nature of the buffer would have, phosphate was substituted for bicarbonate. As shown in Table V, U2′(3′)P is destroyed faster than ψ3′P in potassium phosphate buffer at *p*H 8. As shown in Figure 2, C2′(3′)P, G2′-(3′)P, and A2′(3′)P were also destroyed by periodate oxidation in phosphate buffer at *p*H 7, but at a slower rate than U2′(3′)P. It also appears (Table V) that U2′-

TABLE V: Effect of *p*H on Periodate Oxidation of Pseudouridine-3′-phosphate and Yeast Uridylic Acid.[a]

Starting Material	Time (hr)	Buffer	*p*H	Equivalents CU	Equivalents FoU	Recovered Starting Material (%)	Recovered Ultraviolet-absorbing Product (%)
ψ3′P	23	HCO_3	8.9	0.44	0.28	9	81
ψ3′P	29	HCO_3	7.5	0.23	0	19	42
ψ3′P	27	HCO_3	7.3	0.18	0	34	52
ψ3′P	30	HCO_3	7.0	Trace	0	44	44
U2′(3′)P	23	HCO_3	8.9			66	66
U2′(3′)P	21	HCO_3	7.5			73	73
U2′(3′)P	25	P_i	8.0			25	25
ψ3′P	25	P_i	8.0			69	69

[a] The reaction at *p*H 8.9 was carried out as described in Table I. The other reactions were carried out as follows: An aqueous solution (0.5 ml) containing $NaHCO_3$ (62.5 μmoles), $NaIO_4$ (10.0 μmoles), and ψ3′P (NH_4^+ salt, 1.5 μmoles) or U2′(3′)P (1.5 μmoles) was adjusted to the desired *p*H with CO_2. Aliquots (25 μl) were drawn into capillary tubes. The tubes were sealed, wrapped in aluminum foil, and heated in a water bath at 50°. The progress of the reaction was followed by removing a capillary tube, opening it, and titrating the contents to determine the periodate uptake (see under Experimental). At the times indicated in the table an aliquot was examined by paper electrophoresis (see under Experimental). The products are expressed in equivalents based on the starting material. Recovered ultraviolet-absorbing product is calculated from the sum of the products and the recovered starting material.

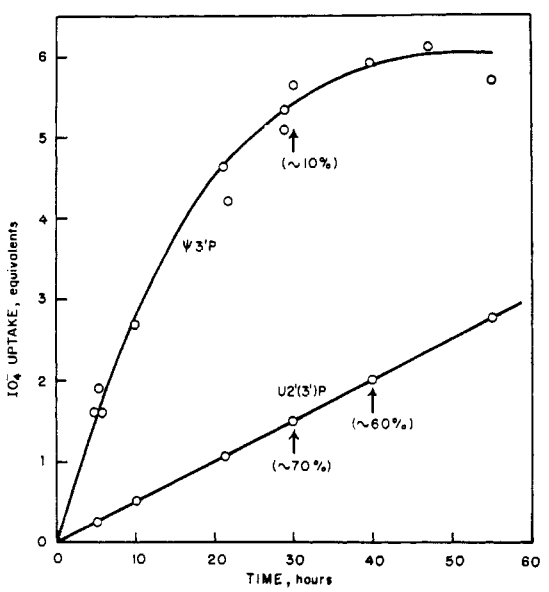


FIGURE 1: Periodate oxidation of pseudouridine-3′-phosphate and uridine-2′(3′)-phosphate at *p*H 7.5 in sodium bicarbonate buffer. The numbers in parenthesis refer to the starting materials remaining at the time indicated (determined by paper electrophoresis; see under Experimental).

(3′)P is destroyed faster in phosphate buffer than in bicarbonate.

Further experiments on the effect of temperature and the ratio of periodate to substrate led to the adoption of sodium bicarbonate buffer, *p*H 7.5, 50°, and 84 moles $NaIO_4$ per mole s-RNA as the most satisfactory conditions for oxidation of s-RNA. It was clear from these model experiments that the chances of achieving a specific cleavage of the pseudouridine residues in s-RNA under these conditions were not good. Nevertheless, as shown in Figures 1 and 2, the rate of oxidation of ψ3′P was considerably faster than the oxidation of other nucleotides and, since the effect of neighboring groups in the polynucleotide chain on the course of the reaction was unpredictable, it seemed worthwhile to attempt the reaction with s-RNA.

Crude s-RNA from *Escherichia coli* was treated under the conditions described, and the course of the reaction was followed by periodate uptake. After 50 hours (30.5 eq periodate taken up),[12] the s-RNA was hydrolyzed with alkali and analyzed for its uridylic and pseudouridylic acid content. The results indicated that 45% of the pseudouridylic acid residues originally present in the s-RNA had disappeared. However, 30% of the uridylic acid residues were also destroyed by the reaction.

In order to see if more extensive oxidation of the pseudouridylic acid residues was possible the reaction was extended to 70 hours using partially purified s-RNA from *E. coli*. During this time 60 eq of periodate was taken up.[12] The results of the analysis on an alkaline hydrolysate of this s-RNA before and

[12] We do not regard the periodate uptake as a valid measure of oxidation of s-RNA. The uptake appeared to increase with time even though no change in the nucleotide analysis of s-RNA could be found.

after periodate oxidation are shown in Table VI. It should be noted that increasing the time of the reaction did not result in any further loss of pseudouridine residues. It is also clear that extensive degradation of other nucleotides occurred.

TABLE VI: Analysis of s-RNA Hydrolysate before and after Periodate Oxidation at pH 7.5 and 50° for 70 Hours.[a]

Nucleotide	Equivalents	
	Control s-RNA	Oxidized s-RNA
C2′(3′)P	18	12
A2′(3′)P	13	10
U2′(3′)P	15	10
G2′(3′)P	24	18
ψ2′(3′)P	2	1.2

[a] See experimental section for details. The equivalents were calculated on the basis of mw = 30,000.

Attempts to demonstrate that cleavage of the polynucleotide chain had occurred were equivocal. Chromatography on DEAE-cellulose showed some small differences between the treated and untreated s-RNA, but no clear-cut separation of any fragments could be achieved.

From these results it seems unlikely that periodate oxidation can be used to split s-RNA specifically. Nevertheless, this work provides compelling evidence in support of the mechanism we have proposed for isomerization of pseudouridine (Chambers *et al.*, 1963) and pseudouridylic acid (Chart 2). All the predictions based on this mechanism have been borne out even though specific cleavage of s-RNA could not be demonstrated. In addition, two unexpected reactions were found: (1) direct oxidation of the carbon attached to the 5 position of uracil, and (2) oxidation of the heterocyclic rings in ψ3′P, U2′(3′)P, C2′(3′)P, and to a lesser extent G2′(3′)P and A2′(3′)P.

The nonspecific oxidation of the heterocyclic rings is an interesting reaction which deserves more attention since there is some indication that similar reactions occur to some extent even under the more normal conditions of periodate oxidation of nucleosides and nucleotides (D. H. Rammler, personal communication).

More recent experiments suggest that it still may be possible to exploit the unusual properties of pseudouridine in other ways to effect a specific cleavage of s-RNA (Tomasz and Chambers, 1964a).

Experimental

Methods and Materials. Uridylic, cytidylic, guanylic, and adenylic acids were mixtures of the 2′ and 3′ isomers. These nucleotides as well as thymine were obtained from Schwarz Bioresearch, Orangeburg, N.Y. 5-Hydroxymethyluracil was obtained from Mann Research Laboratories, New York City. Pseudouridine-3′-phosphate was isolated from yeast RNA (Schwarz Bioresearch) by the method of Cohn (1961). Pseudouridine-3′,5′-diphosphate was synthesized as described in the accompanying paper.

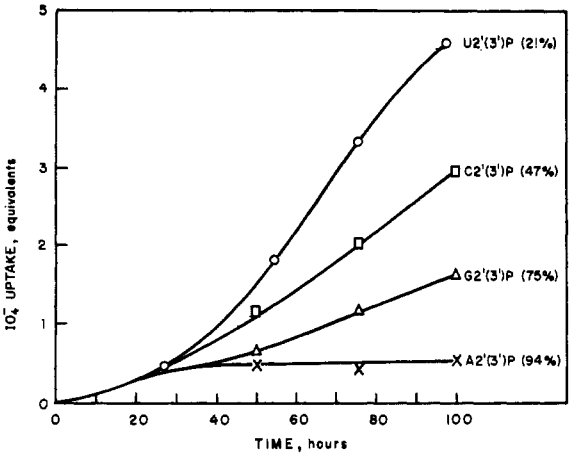


FIGURE 2: Periodate oxidation of yeast nucleotides in phosphate buffer at *p*H 7.0. Yeast nucleotide (1.5 μmoles) and $NaIO_4$ (10 μmoles) were heated at 50° in 0.5 ml of 0.02 M sodium phosphate buffer in a tightly stoppered test tube. Aliquots (25 μl) were removed as indicated for measurement of periodate uptake (see under Experimental). After about 100 hours an aliquot was analyzed by paper electrophoresis at *p*H 8 in 0.067 N sodium phosphate buffer (see under Experimental). The amount of starting material remaining is shown in parentheses. No other ultraviolet-absorbing material was found.

5-Formyluracil. The procedure was modified from that of Cline *et al.* (1959). 5-Hydroxymethyluracil (400 mg, 3.5 mmoles) was dissolved in 40 ml of 0.2 N HCl. Manganese dioxide (4 g) was added to the solution and the mixture was stirred for 2 days at room temperature. Electrophoresis of an aliquot indicated the presence of 5-hydroxymethyluracil, 5-formyluracil, and 5-carboxyuracil. The mixture was filtered and evaporated to dryness *in vacuo*. The residue was redissolved in 84 ml of water, filtered, and made alkaline with a few drops of saturated $NaHCO_3$. The solution was chromatographed on a Dowex 1 (acetate) (8% cross-linked, 200–400 mesh) column (2 × 12.5 cm). Water, 0.02 M acetic acid, and 1% acetic acid eluted 5-hydroxymethyluracil (1.2 mmoles), 5-formyluracil (0.33 mmole), and 5-carboxyuracil (1.2 mmoles), respectively. The fractions containing 5-formyluracil were combined, evaporated to dryness, and recrystallized from water. The crystalline material (28 mg, 0.2 mmole) was homogeneous as judged from paper chromatography and electrophoresis. Its other properties corresponded to those described in the original synthesis (Cline *et al.*, 1959).

5-Carboxyuracil. 5-Carboxyuracil ethyl ester[13] (72 mg) was saponified by refluxing in a mixture of 5% KOH in ethanol (2 ml) and water (2 ml) for 3 hours. The alkali was removed from the solution by addition of Dowex 50 (H^+) (50–100 mesh) ion-exchange resin. The resin was removed by filtration. The filtrate was concentrated *in vacuo* and allowed to stand at room temperature. 5-Carboxyuracil soon crystallized. Its homogeneity was established by paper chromatography (solvent A) and electrophoresis. Its spectral properties agreed with those reported previously (Stimson, 1949).

The amount of *formaldehyde* was determined by the chromotropic acid method as modified by Khym and Cohn (1960). *Inorganic phosphate* was measured by the method of King (1932). *Nucleotides and other ultraviolet-absorbing compounds* were measured spectrophotometrically using the following constants: ψ3'P, ψ3',5'P, $\epsilon_{262}^{2\text{-}7}$ 7900 (Shapiro and Chambers, 1961); FoU, ϵ_{275}^{2} 11,850 (Cline *et al.*, 1959); CU, ϵ_{270}^{3} 11,200 (Stimson 1949); A2'(3')P, ϵ_{260}^{7} 15,000 (Volkin and Cohn, 1954); G2'(3')P, ϵ_{260}^{7} 11,800 (Volkin and Cohn, 1954); U2'(3')P, ϵ_{260}^{7} 9900 (Volkin and Cohn, 1954); C2'(3')P, ϵ_{260}^{7} 7600 (Volkin and Cohn, 1954).

All reaction products were identified by comparison of their ultraviolet-absorption spectra and their chromatographic and electrophoretic properties with authentic samples.

Electrophoresis was carried out on Whatman 3MM paper using an E-C Apparatus Co. unit (Swarthmore, Pa.). Quantitative data were obtained by eluting the ultraviolet-absorbing spots with 0.02 M sodium phosphate buffer, *p*H 7.0, and determining the absorbance in a Beckman DU spectrophotometer against an appropriate paper blank. The mobilities of various compounds are recorded in Table VII.

Paper chromatography was carried out on Whatman No. 40 paper using the descending technique. The R_F values are recorded in Table VII.

Periodate Oxidation. General Remarks. For solubility reasons KIO_4 must be used above *p*H 8. A solution was prepared by dissolving 230 mg of KIO_4 in 2 ml of 1 N KOH and diluting the solution to 7–8 ml. The *p*H was adjusted to about 9.3 with saturated $KHCO_3$ and the volume was adjusted to 10.0 ml. This gave a solution which was 0.1 M in IO_4^- and 0.2–0.25 M in HCO_3^-. The solution was stored in the refrigerator in the dark. Fresh solution was prepared every few days. Below *p*H 8, 0.1 M $NaIO_4$, prepared by dissolving $NaIO_4$ in water, was used. All components of the reaction mixture must contain the same cation as the periodate unless otherwise noted. For reactions at *p*H 7–8, the *p*H was adjusted with CO_2 and the reactions were run in sealed glass tubes. All other reactions were run in tightly stoppered test tubes. All reactions were run in the dark.

Periodate consumption was measured by the Muller-Friedberger procedure[14] slightly modified for small scale: An aliquot (0.05–0.1 ml) of the reaction mixture (containing 0.7–1.4 μmoles IO_4 initially) was added to a mixture of saturated $KHCO_3$ (2 ml) and 20% KI (0.2 ml). The solution was stored in a tightly stoppered test tube in the dark for 10 minutes. The I_2 formed was titrated with 0.0005 N sodium arsenite (used as a primary standard).

Occasionally, the spectrophotometric procedure of Dixon and Lipkin (1954) was used. An aliquot (25 μl) of the reaction mixture containing about 0.35 μmole KIO_4 initially was diluted to 5 ml with 0.025 M potassium phosphate buffer, *p*H 11, in order to prevent precipitation of KIO_4. When $NaIO_4$ was used this step was unnecessary.

In the experiment described in Table I, periodate consumption was measured both by the Muller-Friedberger procedure and by the Malaprade procedure.[14] The results compared favorably (4.32 versus 4.62 eq).

5-Hydroxymethyluracil was oxidized as follows: Hydroxymethyluracil (64 μmoles) was mixed with 190 μmoles KIO_4 in 10 ml of 0.2 M $KHCO_3$. The mixture was heated for 10 hours at 50°. An aliquot of the reaction mixture was examined by ion-exchange chromatography, using the conditions described in Table I. The results were: starting material 3.0 μmoles, FoU 1.39

TABLE VII: Electrophoretic and Chromatographic Mobilities.

Compound	R_u[a]	R_F in Solvent A[b]	B[c]	C[d]	D[e]
ψ		0.36			
ψ2'P	14.9	0.26	0.29		0.11
ψ3'P	14.9	0.26	0.27		0.11
ψ2',3' cyclic P					0.31
ψ3',5'DP	21.5	0.12			
Formyluracil	8.0		0.68	0.62	0.62
Carboxyuracil	13.2			0.36	0.11
Carboxyuracil ethyl ester	2.5				
Hydroxymethyluracil				0.76	0.54

[a] Electrophoresis; 0.05 M sodium phosphate buffer, *p*H 7.5; 1000 v for 2 hours; R_u is the mobility in cm relative to uracil. [b] 1-Propanol–concd NH_4OH–H_2O (11:7:2, v/v/v). [c] Isobutyric acid–0.5 M NH_4OH (10:6, v/v, *p*H 3.7). [d] Ethanol–1 M ammonium acetate (7.5:3, v/v). [e] 2-Propanol–concd NH_4OH–H_2O (7:1:2, v/v/v).

[13] We are indebted to Dr. Jack Fox for a sample of this material.

[14] For a critical discussion of various methods for determining periodate consumption see Dyer (1956). The Fleury-Lange method gave variable results in our hands, probably owing to the small scale, and was unusable.

μmoles, CU 0.5 μmole. The loss of ultraviolet-absorbing material was not established quantitatively.

Thymine was oxidized as follows: Thymine (8.6 μmoles) was mixed with KIO_4 (56 μmoles) in 2.76 ml of 0.2 M $KHCO_3$. The solution was heated at 50° for 42 hours. Ion-exchange chromatography (as described in Table I) yielded a large fraction with water. This proved to be a mixture of thymine (R_F 0.66) and 5-hydroxymethyluracil (R_F 0.42) in a ratio of 4.3:1 as determined by paper chromatography (butanol–acetic acid–water, 4:1:5, v/v/v). A small amount of FoU was eluted with 0.02 M acetic acid.

s-RNA was oxidized as follows: Saturated $NaHCO_3$ (0.5 ml) and 0.1 M $NaIO_4$ (1.0 ml) were mixed and diluted with 1.0 ml of water. The *p*H was adjusted to 7.5 with CO_2. Partially purified *E. coli* phenylalanyl s-RNA (*ca.* 5 mg) was dissolved in about 0.4 ml of water, and the *p*H was adjusted to 7.5 with 1 N NaOH. An aliquot (0.3 ml) containing 85 A_{260} units (*ca.* 4 mg) was removed and mixed with 0.3 ml of the buffered $NaIO_4$ solution. The mixture was heated in a tightly stoppered test tube at 50° for 75 hours in the dark. Sixty equivalents of sodium periodate was taken up during this time.[12] An aliquot (0.4 ml) of the reaction mixture was removed after 75 hours and dialyzed against water. The solution was then evaporated to dryness under reduced pressure. The residue was dissolved in 2 ml of 0.5 N NaOH and heated at 37° for 20 hours. Amberlite IR-120 (H^+) ion-exchange resin was added in small amounts to the hydrolysate until the *p*H fell to 7.0. The resin was removed by filtration and the filtrate was fractionated on a column of Dowex 1 (formate) ion-exchange resin (200–400 mesh, 8% cross-linked, 0.5 × 9 cm). The nucleotides were eluted as described by Cohn (1960, his Figure 1). The ψ2′(3′)P fraction was concentrated to dryness under reduced pressure and the residue was redissolved in water. The aqueous solution was passed through a small Amberlite IR-120 (H^+) column and evaporated to dryness again. The residue, now free from formic acid and formate salts, was redissolved in water. The ultraviolet spectra indicated that the pseudouridylic acid was contaminated by some other ultraviolet-absorbing material. The amount of pseudouridylic acid present was calculated by assuming that it was the only component present which absorbed significantly at 300 mμ at *p*H 12, and using the molar extinction coefficient $\epsilon_{300}^{12} = 3.2 \times 10^3$ (calculated from the ultraviolet-absorption spectrum of pseudouridine: R. Shapiro and R. W. Chambers, unpublished data). The amounts of the other nucleotides were determined by pooling the appropriate fractions and determining their total absorbance at λ_{max}. The ϵ_{max} values given were used to calculate the molar ratios shown in Table VI.

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