during the processing of the wheat germ and/or the preparation of the RNA.

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The Chemistry of Pseudouridine. Synthesis of Pseudouridine-5′-Diphosphate*

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The synthesis of 5-β-D-ribofuranosyluracil-5′-diphosphate (pseudouridine-5′-diphosphate) has been achieved from 5-β-D-ribofuranosyluracil (pseudouridine) using the cyanoethylphosphate method to prepare the monophosphate and the nucleoside phosphoramidate method to make the diphosphate. During the synthesis of the monophosphate two side reactions occurred. One of these was an alkylation of the pyrimidine ring to give a compound tentatively assigned the structure of 1-*N*-cyanoethyl-5-β-D-ribofuranosyl-5′-phosphate. The other involved an anomerization of the C—C glycosyl bond to give 5-α-D-ribofuranosyluracil-5′-phosphate. This anomerization reaction also occurred in the preparation of 5-β-D-ribofuranosyluracil-5′-phosphoramidate and probably in the synthesis of 5-β-D-ribofuranosyluracil-5′-diphosphate. The ultraviolet spectra of all the 5′-substituted derivatives of pseudouridine resemble that of 5-α-D-ribofuranosyluracil (pseudouridine-B) *regardless of the configuration of the glycosyl bond.* It is postulated that the spectral difference at *p*H 12 between various isomers and derivatives of pseudouridine is due to the presence or absence of specific hydrogen bonds. The role of the allyl ether grouping in pseudouridine chemistry is discussed. Tentative structural assignments for the pseudouridine isomers (B, C, A_F, and A_S) are made on the basis of spectral data and a consideration of reaction mechanisms.

As an extension of our studies on the specificity of polynucleotide phosphorylase (Lengyel and Chambers, 1960) and on the chemistry of pseudouridine (Shapiro and Chambers, 1961) we have undertaken the synthesis of ψUDP.[1] The reaction sequence employed is outlined in Figure 1. These reactions are all well known and in fact the pathway is identical to that employed for the synthesis of 2-thiouridine-5′-diphosphate (Lengyel and Chambers, 1960). However, certain special problems were anticipated in applying these reactions to pseudouridine and others were encountered during the course of the work described in this paper.

The first problem was a source of starting material. The isolation procedures from ribonucleic acid (Yu and Allen, 1959; Cohn, 1960, 1961) are not easily adaptable to the preparation of gram quantities of pseudouridine, and the only published synthesis (Shapiro and Chambers, 1961) is not satisfactory as a preparative method because of the low yield. Reports by Adler and Gutman (1959) and by Adams *et al.* (1960) suggested that human urine was a reasonably good source. In collaboration with Dr. W. E. Cohn, a satisfactory

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[1] The following abbreviations are used: ψUMP, pseudouridine-5′-monophosphate; ψUMP-NH_2, pseudouridine-5′-phosphoramidate; ψUDP, pseudouridine-5′-diphosphate.

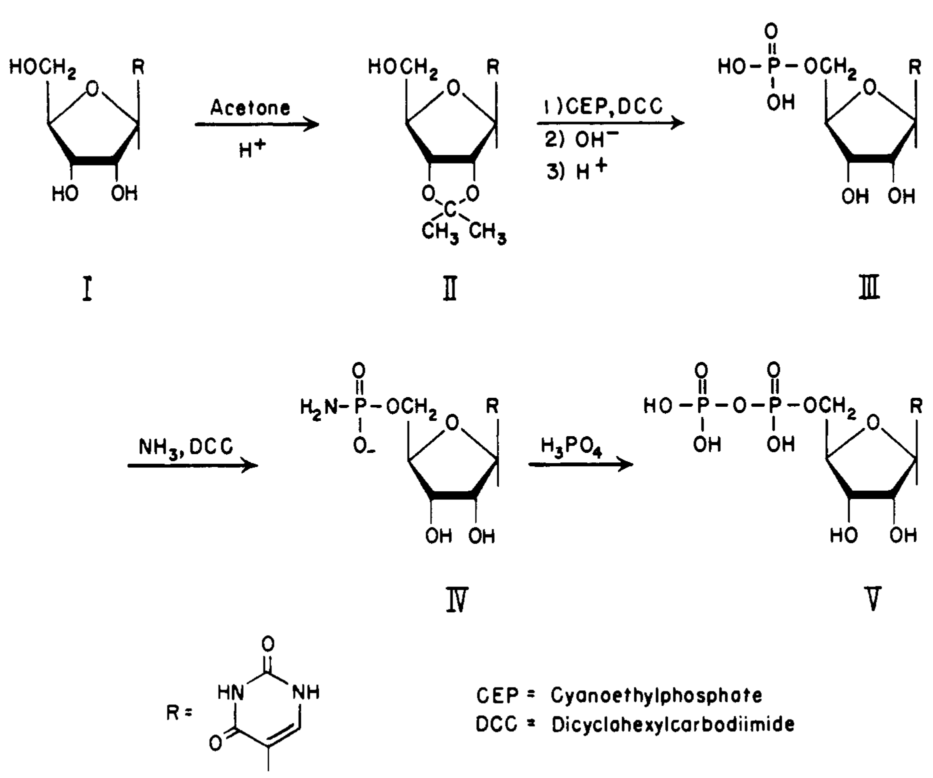


Fig. 1.—Synthesis of pseudouridine-5'-diphosphate.

though tedious procedure was developed which yielded 300–400 mg of pseudouridine per 10 liters of urine.

The second problem concerned possible isomerization of pseudouridine during the synthesis of ψUDP. It has been shown (Cohn, 1960) that pseudouridine is converted to a mixture of four isomers, designated A_F, A_S, B, and C, when it is heated in acid. There is also evidence that these same isomers are formed in alkali (Cohn, 1961). The structures of these isomers have been tentatively assigned as follows: A_F, 5-α-D-ribopyranosyluracil; A_S, 5-β-D-ribopyranosyluracil; B, 5-α-D-ribofuranosyluracil; C, 5-β-D-ribofuranosyluracil (Shapiro and Chambers, 1961). Since each step in the synthesis of ψUDP involved either acidic or alkaline conditions, or both, isomerization of the starting material (C isomer) was a distinct possibility.

Conversion of pseudouridine to 2',3'-*O*-isopropylidene pseudouridine (II, acetone pseudouridine) could be accomplished quantitatively on a very small scale (100 absorbance units at 260 mμ) by simply shaking pseudouridine with acetone and Dowex-50-H$^+$ ion-exchange resin. However, this procedure was unsatisfactory as a preparative method, even on a 10-mg scale, because of the poor solubility of pseudouridine in acetone. Use of *p*-toluenesulfonic acid as a catalyst (Hampton and Magrath, 1957) gave better results, but a considerable amount of unchanged starting material remained after 24 hours, again because of poor solubility of the starting material. The most satisfactory method utilized di-*p*-nitrophenylphosphoric acid as a catalyst in the presence of dimethoxypropane to remove the water formed during the reaction (Hampton, 1961). Under these conditions crystalline acetone pseudouridine was obtained in 78% yield.

Since acidic conditions were used in this step, isomerization of pseudouridine-C to produce the acetone derivative of A_F, A_S, and B was possible. The acetone derivatives of A_F and B are easily distinguishable from the acetone derivative of C on the basis of their ultraviolet spectra (Cohn, 1960) and our spectral data on acetone pseudouridine showed no evidence of these isomers. However, if they were present in very small amounts they might have escaped detection. Furthermore, A_S can not be distinguished from C in this way. An attempt was made to detect the presence of A_S by hydrolyzing acetone pseudouridine with 0.01 N HCl for 24 hours at room temperature. The resulting pseudouridine was then fractionated by ion exchange (Cohn, 1960; Shapiro and Chambers, 1961) and a small amount of material (8%) was found which appeared to be A_S. Another small peak in the position normally occupied by the B isomer was also found, but its spectrum at *p*H 12 did not resemble B or any other known pseudouridine isomer. This material is still unidentified. This procedure was, of course, equivocal because acidic conditions were used to remove the acetone groups and isomerization may well have occurred at this step rather than during the synthesis.

On reinvestigation of this point after completion of the ψUDP synthesis, we found it was possible to separate another component (~10%) from acetone pseudouridine-C by ion-exchange chromatography (see under Experimental). This material emerged from the column in a position corresponding roughly to the A isomers of pseudouridine. Paper chromatography in two solvents resolved this material into two compounds, one with an R_F identical to that of acetone pseudouridine-C and one slightly lower. The slower material was periodate positive indicating the presence of a vicinal glycol, while the faster spot gave a negative test. The spectra of both were very similar to pseudouridine-C (or A_S). The structure of these components cannot be definitely assigned at this time although most of the data are consistent with acetone pseudouridine-A_S, and pseudouridine-A_S. It should be borne in mind that these impurities were present in the following phosphorylation step.

Acetone pseudouridine was phosphorylated by the cyanoethylphosphate procedure (Tener, 1961). Paper chromatography indicated the reaction was complete

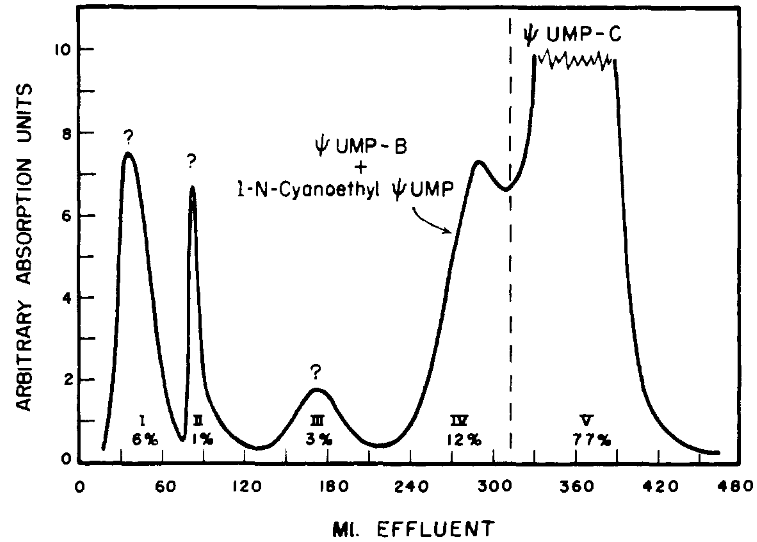


Fig. 2.—Fractionation of pseudouridine-5′-phosphate reaction mixture by ion exchange (Dowex-1-formate, linear gradient elution with formic acid, see under Experimental for details).

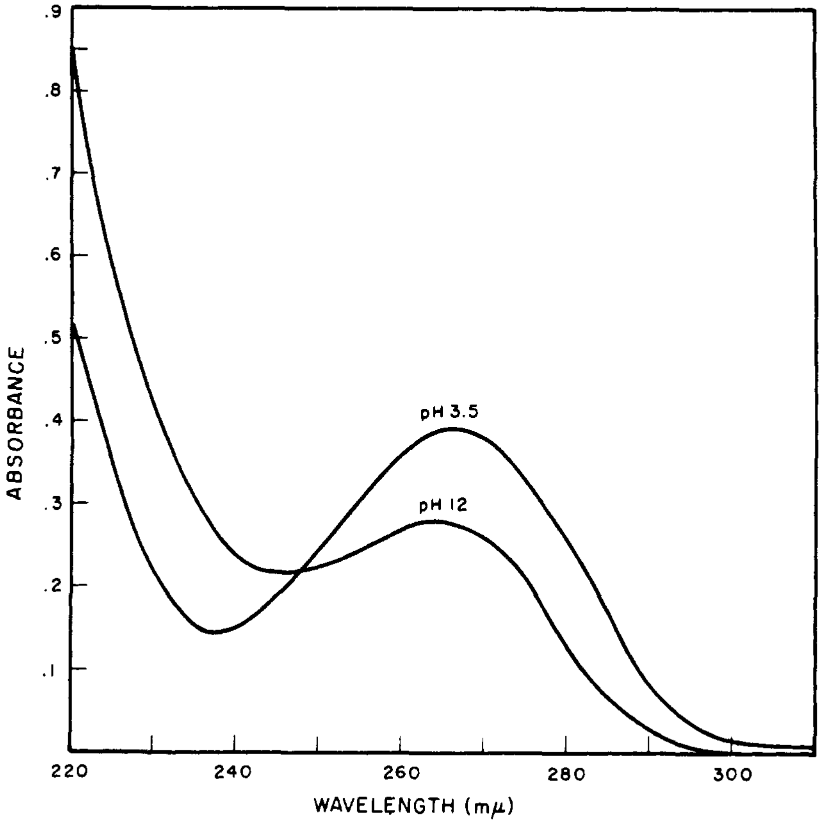


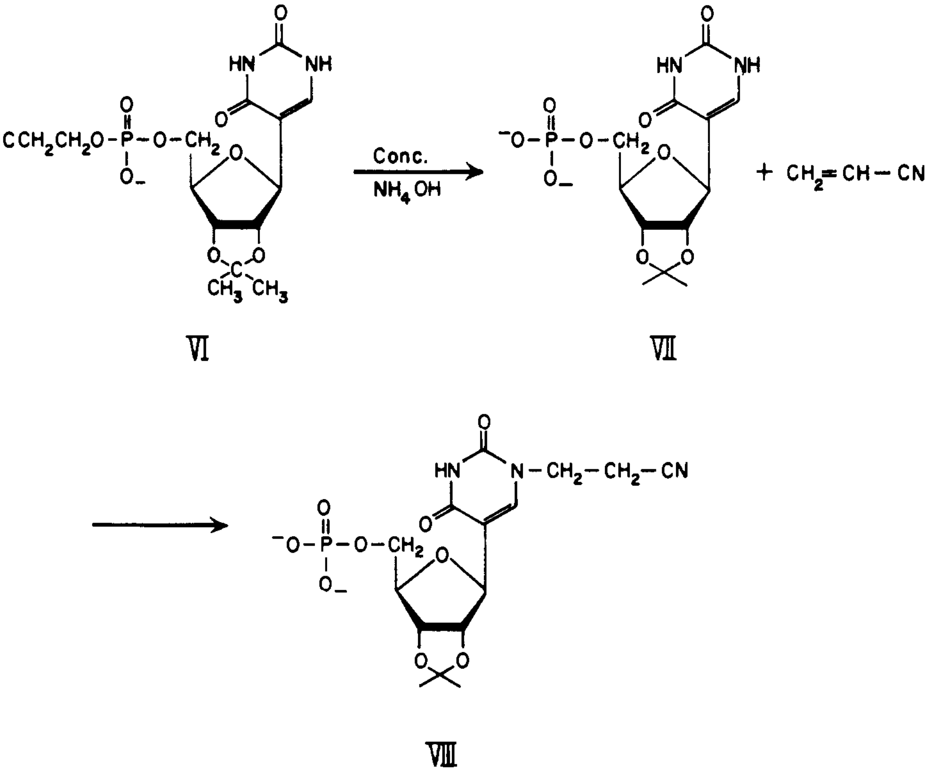
Fig. 3.—Spectra of *N*-cyanoethyl pseudouridine.

after 3 days at room temperature. During the removal of the solvent partial hydrolysis of the acetone groups apparently occurred, giving a new chromatographic spot whose properties were consistent with pseudouridine-5′-cyanoethylphosphate. Considering the mildness of the conditions, this is a surprising result.

Removal of the cyanoethyl groups was complete in 1 hour in concentrated ammonium hydroxide at 75°. This step was followed, without isolation, by acid hydrolysis to remove the remaining acetone groups.

Since the phosphorylation was carried out in pyridine the chances for isomerization were small. Removal of the blocking groups required both hot acid and hot alkali. Therefore isomerization was a distinct possibility in these steps. However, once the 5′-hydroxyl group is esterified, pyranose formation is impossible. Thus, if isomerization occurred and if the structures of the pseudouridine isomers had been assigned correctly, only furanose forms (B and C) could be present. To test this the entire reaction mixture (after removal of the blocking groups) was fractionated by ion-exchange chromatography. The results are shown in Figure 2. The first three peaks (6%, 1%, and 3%) were not identified. Peak V (77%) was identified as ψUMP-C by its spectra. Rechromatography by ion exchange indicated that it was the pure C isomer.

Material from peak IV (12%) was not degraded by crude snake venom and phosphate was released only



VI VII VIII

FIG. 4.—Postulated formation of 1-*N*-cyanoethylpseudouridine-5′-phosphate.

very slowly by tobacco phosphatase. ψUMP-C, on the other hand, was rapidly degraded by both of these enzyme preparations. Chromatography on paper in isopropyl alcohol–ammonium sulfate revealed that peak IV contained two periodate-positive nucleotides having R_F values of 0.36 and 0.45 (solvent B), respectively. The two materials were isolated by paper chromatography and converted to their corresponding nucleosides with tobacco phosphatase.

The nucleoside derived from R_F 0.36 material was identified as pseudouridine-B by its ultraviolet spectra and by ion-exchange chromatography. The nucleoside derived from R_F 0.45 material gave the spectra shown in Figure 3. The absence of a bathochromic shift at *p*H 12 compared to *p*H 3.5 showed that N^1 did not have a dissociable hydrogen (Shugar and Fox, 1952; Cohn, 1960). Consideration of the method of synthesis suggested that alkylation of N^1 by acrylonitrile had occurred to give VIII as shown in Figure 4. Some precedence for this reaction is found in the alkylation of isocytosine by acrylonitrile in strong alkali to give 1-*N*-carboxyethylisocytosine (Angier and Curran, 1961). Further evidence for this structure was obtained by alkaline hydrolysis to convert the nitrile to a carboxylic acid. Paper electrophoresis showed that a new compound with one additional negative charge had indeed been formed. Finally, heating some pseudouridine with acrylonitrile in concentrated ammonium hydroxide gave the same nucleoside in addition to some material which was assumed to be the 1,3-*N*-dicyanoethylpseudouridine.

ψUMP-C isolated from peak V was used for the synthesis of ψUMP-NH_2 using our standard reaction conditions (Chambers and Moffatt, 1958). This step also requires heating with ammonium hydroxide so that isomerization to the B isomer was possible. Therefore the crude reaction mixture was fractionated on Dowex-1-HCO_3^- using a stepwise elution program with increasing concentrations of ammonium bicarbonate. The elution pattern is shown in Figure 5. The materials in fractions I, III, and IV were not identified. Fractions V and VI were identified as ψUMP-B and ψUMP-C, respectively. Fraction II contained ψUMP-NH_2. At this time we were unable to separate any ψUMP-NH_2-B by ion exchange.[2] However, the presence of B isomer was strongly indicated by the identification of ψUMP-C and ψUMP-B in fractions V and VI. Therefore material from fraction II was treated with crude snake venom. Eighty per cent of the ψUMP-NH_2 was hydrolyzed to pseudouridine-C. The remaining ψUMP-NH_2 was not hydrolyzed further even when additional venom was added. Hydrolysis of this resistant material with 0.01 N HCl for 5 hours at room temperature gave ψUMP which was converted to pseudouridine with tobacco phosphatase. This material was identified as pseudouridine-B. Thus, the crude ψUMP-NH_2 was about 80% C isomer and 20% B isomer.

Since we were unable to fractionate the ψUMP-NH_2 isomers at this time, the crude mixture was used for the preparation of ψUDP. After the amidate was treated with anhydrous phosphoric acid in the usual manner (Chambers *et al.*, 1960) the reaction mixture was fractionated on Dowex-1-HCO_3^-. The elution pattern is shown in Figure 6. Here a clean separation of ψUMP-NH_2-B, ψUMP-NH_2-C, ψUMP-B, and ψUMP-C can be seen. The ψUDP isomers are seen as two incompletely resolved peaks. Since our starting material contained ψUMP-NH_2-B, fraction IVa was almost certainly ψUDP-B. This was confirmed by hydrolysis with snake venom to pseudouridine-B. The hydrolysis was very slow and no intermediate ψUMP was detected. The material in fraction IVc was rapidly cleaved to pseudouridine-C by snake venom.

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[2] Later work on the fractionation of ψUDP did lead to a fractionation procedure which would separate the B and C isomers of ψUMP-NH_2. See under Experimental, and Fig. 6.

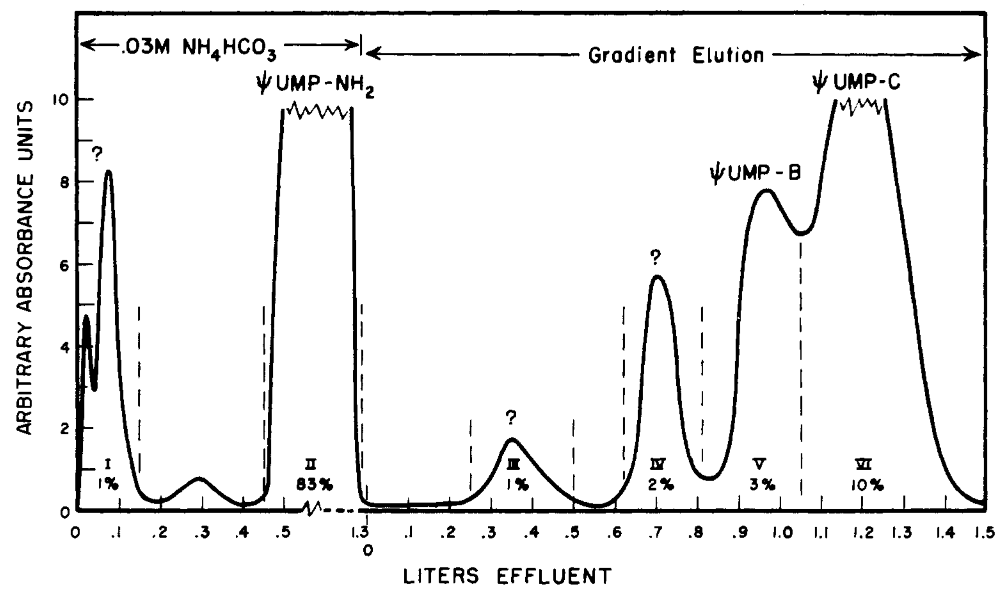


FIG. 5.—Fractionation of pseudouridine-5′-phosphoramidate reaction mixture by ion exchange (Dowex-1-HCO$_3$).

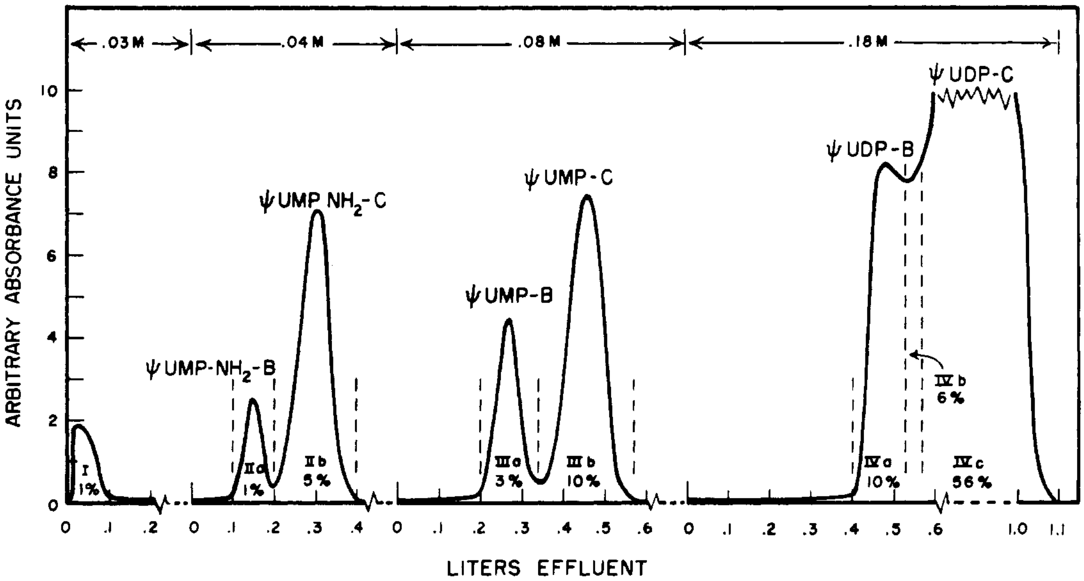


FIG. 6.—Fractionation of pseudouridine-5′-diphosphate reaction mixture by ion exchange (Dowex-1-HCO$_3$, elution with NH$_4$HCO$_3$).

DISCUSSION

Some Comments on the Synthesis.—Some puzzling discrepancies exist between our observations on the synthesis of ψUMP and those reported by Goldberg and Rabinowitz (1961) using the same method. They reported the formation of dipseudouridine phosphate. Presumably, this is a 5′,5′-diester since the 2′- and 3′- positions were blocked in the synthesis. We did not find any such material although a very small peak (peak III in Figure 2) of unidentified material was observed in a position which could correspond to that described by the above authors (no experimental details are reported). On the other hand, they did not report any ψUMP-B or 1-*N*-cyanoethyl ψUMP.

The formation of the 1-*N*-cyanoethyl derivative is particularly interesting since this type of reaction has not been reported before with the cyanoethylphosphate method. In other nucleotides, where N^1 participates in the *N*-glycoside bond, N^1 is not available for this type of side reaction. Apparently N^3 is not reactive enough to form any significant amount of the 3-*N*-cyanoethyl derivative under the conditions used. It should be emphasized that a reaction does occur with N^3 under suitable conditions. Thus when pseudouridine was treated with *excess* acrylonitrile, 1-*N*-mono- and 1,3-*N*-dicyanoethylpseudouridine were formed, but no 3-*N*-monocyanoethylpseudouridine was found.

Finally, some comments on the yield in this synthesis are appropriate. In our experience with synthesis of several different nucleotides the cyanoethylphosphate method gives yields of 75–85% (isolated material) and is by far the most satisfactory general method for preparation of 5′-nucleotides. The major source of difficulty is incomplete removal of blocking groups, but this is easily solved by pilot runs on a small scale to find the optimum conditions. The nucleoside phosphoramidates are obtainable in 75–95% yield (isolated) and they are converted to the corresponding nucleoside diphosphates in 60–85% yield (isolated). An over-all yield of about 25% can be expected starting from the nucleoside.

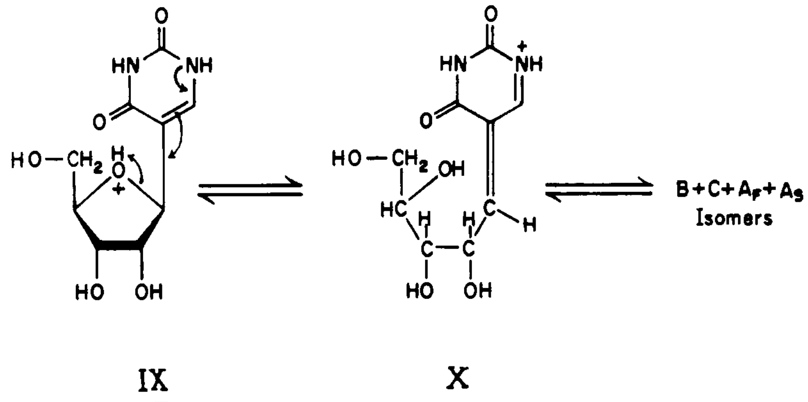


FIG. 7.—Isomerization of pseudouridine in acid.

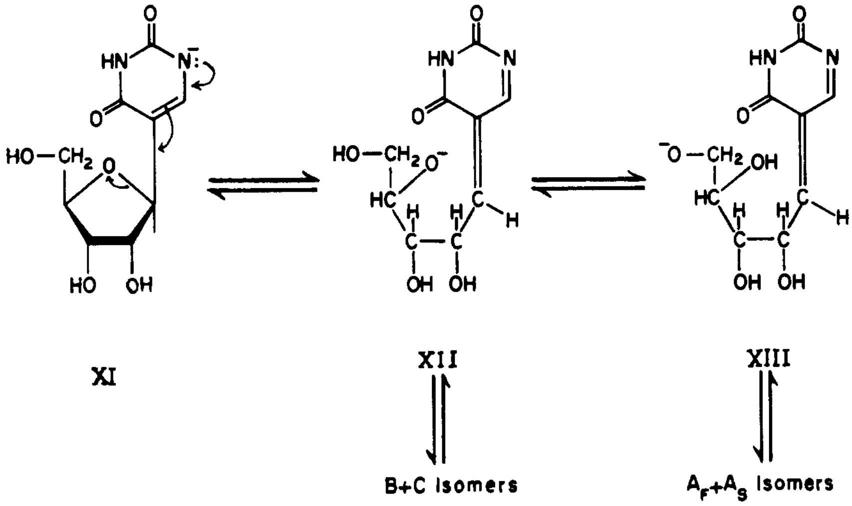


FIG. 8.—Isomerization of pseudouridine in alkali.

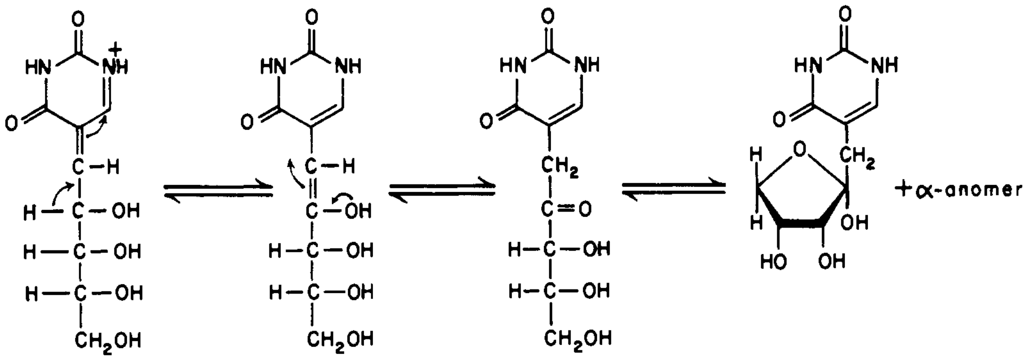
Similar yields were experienced with pseudouridine if isomerization is neglected. However, since most workers are interested only in the natural, β-furanose form, the isomerization cannot be discounted in considering over-all yields. Since a mixture of ψUMP-NH_2-B and C was used in the last step we do not know whether isomerization occurs here. However, isomerization definitely occurs in the two preceding steps and possibly in the first step as well. In the later steps the distribution of B and C isomers was about 1:4. Thus a 20% loss in natural isomer occurs at each step if purification is carried out prior to continuing. This loss can be minimized by using an unfractionated mixture of both isomers in each step and separating the ψUDP isomers at the end. In this way an over-all yield of 15–20%, starting from pseudouridine-C, is to be expected.

Isomerization of Pseudouridine.—Cohn (1960) has shown that an equilibrium mixture of pseudouridine isomers (A_S, A_F, B, and C) is produced when any one of them is heated in acid. It was also reported that B, C, and A_S, but not A_F, were produced when pseudouridine-3'-phosphate was heated in alkali (Cohn, 1960). Recently we have shown that A_F, A_S, and B are produced when pseudouridine-C is heated in alkali although only small amounts of A_F are formed under the conditions studied so far (V. Kurkov and R. W. Chambers, unpublished observations).

Before considering the chemistry of pseudouridine in some detail and attempting to reach some conclusions about this interesting isomerization, several experimental observations and conclusions should be recalled. (1) The predicted β-furanosyl structure for pseudouridine-C (Shapiro and Chambers, 1961) has been confirmed experimentally (Michelson and Cohn, 1962). (2) Both B and C take up one mole of periodate while A_S and A_S take up two moles and release 1 mole of acid (Cohn, 1960). No formaldehyde is formed by any of these isomers. (3) The ultraviolet spectra at pH 12 of A_S and C are similar. The ultraviolet spectra of A_F and B at pH 12 are similar to each other and different from those of A_S and C. (4) Pseudouridine-C gives an "abnormal" orcinal color with a peak at 515–535 $m\mu$ (Cohn, 1960). (5) On the basis of nuclear magnetic resonance data Cohn (1960) has concluded that the ". . . various forms of pseudouridine involve conformational or chemical changes in the ribose component only." (6) A_F and A_S run close together and are difficult to separate on chloride-borate ion-exchange columns (Cohn, 1960; Shapiro and Chambers, 1961). B and C emerge from these columns later and are easily separated.

The existence of a RO—$\overset{|}{\text{C}}$H—CH=$\overset{|}{\text{C}}$— grouping in pseudouridine has been pointed out by Cohn (1960). This type of grouping also occurs in benzyl and allyl ethers. The tendency of these compounds to undergo hydrogenolysis of the ether linkage rather than hydrogenation of the double bond is well known (Hartung and Simonoff, 1953) and it is not surprising that pseudouridine undergoes a similar reaction to give the H-isomer (Cohn, 1960).

This allylic system would also be expected to isomerize in acid as shown in Figure 7. The first step is protonation of the sugar ring oxygen to give IX. Ring opening gives the intermediate X which can undergo



XIV XV XVI

FIG. 9.—Postulated Amadori-type rearrangement of pseudouridine.

ring closure in four ways to give two furanose and two pyranose forms.

This mechanism is analogous to that proposed by Kenner (1957) for acid hydrolysis of glycosylamines, including nucleosides. The difference lies in the fact that ring opening in a glycosylamine produces a Schiff base which undergoes hydrolysis so that re-formation of the starting material or isomerization is not detected. With pseudouridine we are dealing with a stable carbon-carbon bond and hydrolysis does not occur so that re-formation of starting material or isomerization is observed.

A similar isomerization would be expected in alkali (Fig. 8) except that the driving force is removal of the *N*-1 proton to give XI, which ring opens to XII. Ring closure of XII would give α- and β-furanosyl anomers. One would also expect proton exchange to occur giving XIII (as well as a neutral species with protons on both the 4′- and 5′-oxygens). Ring closure of XIII would give α- and β-pyranosyl anomers.

These mechanistic considerations as well as the data cited above were the basis of our previous structural assignments (Shapiro and Chambers, 1961). Now that the structure of pseudouridine-C has been definitely established (Michelson and Cohn, 1962) there is little doubt that pseudouridine-B is 5-α-D-ribofuranosyluracil.

The available data and the mechanisms are also consistent with assignment of A_F and A_S as 5-α- and 5-β-D-ribopyranosyluracil, respectively. However, these are not the only structures for A_F and A_S which are consistent with the observed data. An alternative possibility was recognized when we tried to explain the orcinol color produced by pseudouridine.

TABLE I
SPECTRAL GROUPS OF PSEUDOURIDINE AND ITS DERIVATIVES

Group I	A_{280}/A_{260} at pH 12	Group II	A_{280}/A_{260} at pH 12
Uracil[a]	1.4	5-Hydroxymethyl-uracil[a]	1.8
Thymine[a]	1.3		
Pseudouridine-B[a]	1.5	Pseudouridine-C[a]	2.0
Pseudouridine-A_F[a]	1.4	Pseudouridine-A_S[a]	2.5
Pseudouridine-H[a]	1.5	Acetone pseudo-uridine-C[b]	2.1
5′-ψUMP-C	1.4		
5′-ψUMP-B	1.3	Acetone cyclo-pseudouridine[b]	3.1
5′-ψUMP-NH_2-C	1.6		
5′-ψUMP-NH_2-B	1.3	2′-ψUMP-C	1.9
5′-ψUDP-C	1.3	3′-ψUMP-C	1.9
5′-ψUDP-B	1.3		

[a] Cohn (1960). [b] Michelson and Cohn (1962).

According to Cohn (1960), when the orcinol reaction is carried out on pseudouridine a blue-green is produced. This color is characteristic of certain keto sugars (among other compounds) rather than ribose. Cohn considers the attachment of C-1′ to the sugar ring oxygen of critical importance in the color reaction because the H-isomer, in which this bond is missing, does not give any orcinol color. We disagree with this interpretation. The attachment of C-1′ to the sugar ring oxygen probably has nothing to do with the chromophoric group.

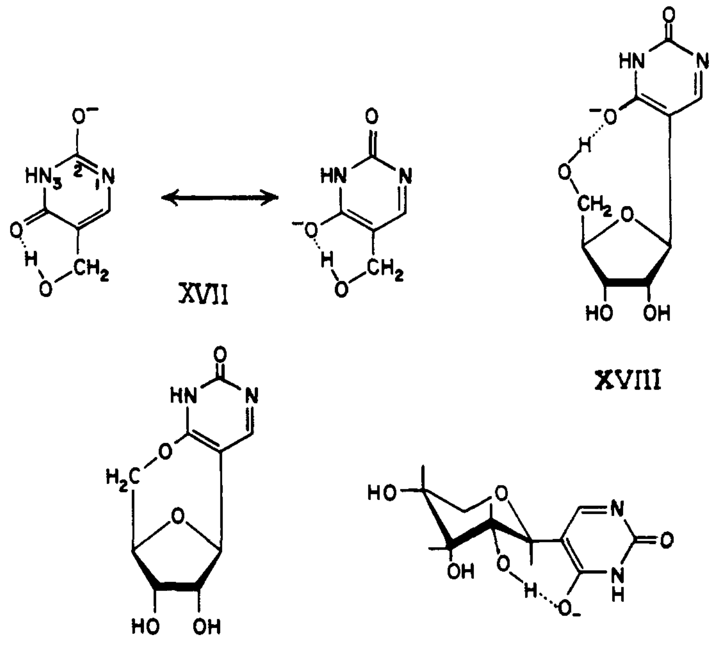
We postulate that under the conditions of the orcinol reaction, the pseudouridine ring opens to give XIV (Fig. 9), and this intermediate undergoes an Amadori-type rearrangement to give the 1′-deoxy-2-keto derivative XV. Pseudouridine-H, which cannot undergo this type of rearrangement, should not give an orcinol color.

The possibility that an Amadori rearrangement of pseudouridine may occur complicates the assignment of structure to the A isomers. Cohn recognized that an isomer analogous to XV might exist, although he gave no explanation of how it could form. He also pointed out that such an acyclic structure was difficult to reconcile with the periodate data. However, this compound (XV) would be expected to cyclize giving a mixture of furanosyl anomers (XVI). This cyclic structure would be consistent with the periodate data.

The argument that the A isomers are formed in alkali and the Amadori rearrangement does not normally proceed in alkali is not valid in this case. Rearrangement of glycosylamines in alkali does not go because the intermediate equivalent to XIV does not form under these conditions. Once such an intermediate does form, as in the case of pseudouridine, rearrangement can take place as readily in alkali as in acid.

Thus, there are at least four reasonable structures which can be assigned to the A isomers. It is somewhat difficult to rationalize the behavior of the pseudouridine isomers on borate ion-exchange columns if A_F and A_S are assigned the furanosyl forms XVI. For this reason we still favor the assignment of A_F and A_S as pyranosyl anomers (Shapiro and Chambers, 1961). However, more experimental data is clearly needed before any structures can be assigned to A_F and A_S with assurance.

The Ultraviolet Spectra of the Pseudouridine Isomers and Their Derivatives.—The spectra of all pseudouridine isomers and their derivatives with a dissociable hydrogen at N-1 show λ_{max} values in the 285–288 mμ region



XVII XVIII XIX XX

FIG. 10.—Proposed hydrogen bonds in some 5-substituted uracil derivatives.

when this proton is removed (*ca.* pH 12). However, the shapes of the spectral curves at pH 12 fall clearly into two groups: those showing a symmetrical peak and an A_{280}/A_{260} at pH 12 similar to 5-hydroxymethyluracil[3] and those showing a skewed or broadened peak and an A_{280}/A_{260} at pH 12 similar to uracil and thymine. The compounds in each class are shown in Table I.

The pK values for dissociation to monoanion for uracil (9.5), thymine (9.9), and 5-hydroxymethyluracil (9.8) are similar (Levene *et al.*, 1926; Cohn, 1960). The next dissociation is above pH 13. Furthermore, the A_{280}/A_{260} ratios for these compounds and the pseudouridine isomers are constant between about pH 11.8 and 12.3 (Cohn, 1960), indicating that each compound is present as a monoanion at pH 12. Thus, we are faced with the task of explaining why thymine and 5-hydroxymethyluracil have different spectra at pH 12 and why pseudouridine-B behaves like thymine while pseudouridine-C resembles 5-hydroxymethyluracil even though these pseudouridine isomers differ only in the configuration of the glycosyl bond.

Thymine (group I) and 5-hydroxymethyluracil (group II) differ only in the nature of the 5-substituent. Inspection of molecular models shows that 5-hydroxymethyluracil can form a hydrogen bond between the 5-hydroxymethyl group and the 4-carbonyl group of the ring (Fig. 10, XVII). Thymine obviously cannot form such a hydrogen bond. We postulate that (1) group II compounds have a hydrogen bond between a sugar hydroxyl and the pyrimidine 4-carbonyl while group I compounds do not have such a bond, and that (2) the spectral differences between these two groups at pH 12 are due to this hydrogen bond.

Similar spectral data have been used previously for predicting the position of the phosphate group in pyrimidine nucleotides (Fox *et al.*, 1953). In this case, the spectral shifts which occurred between 0.01 M and 1 M NaOH were interpreted as the effect of rupturing a hydrogen bond between the 2′-hydroxyl and 2-carbonyl of cytidine and uridine derivatives. Although spectral shifts which could be correlated with a 2-5′ interaction were noted by Fox and his coworkers, these effects were regarded as minor because cytidine-2′ phosphate showed no spectral shift between pH 12 and 14.

A molecular model of pseudouridine-C (β-furanosyl) shows that a hydrogen bond is possible between the 4-carbonyl and *either* the 2′- or the 5′-hydroxyl groups. The similarity in the spectra of pseudouridine and acetone pseudouridine (Michelson and Cohn, 1962) in which the H-bond to the 2′-hydroxyl is not possible suggests that the 4-5′-interaction (Fig. 10, XVIII) predominates in the furanosyl form.

This conclusion is supported by the spectra of the pseudouridine derivatives which are phosphorylated on the 5′-position. Here 4-5′-interaction is no longer possible and *all* these compounds belong to group I. Similarly, models of pseudouridine-B (α-furanosyl) show that a hydrogen bond from either the 2′- or the 5′-hydroxyl to the 4-carbonyl is unlikely. The B-isomer, as expected, belongs to group I. Phosphorylation of pseudouridine-C on either the 2′- or the 3′-hydroxyl should have no effect on the 4-5′-H bond and, as expected, both these compounds belong to group II.

Cyclopseudouridine (Fig. 10, XIX) (Michelson and Cohn, 1962) represents an extreme case of 4-5′-interaction in which a covalent bond instead of a hydrogen bond is involved. As expected, it not only belongs to group II, it shows the largest difference from group I compounds with respect to A_{280}/A_{260}.

If our interpretation of spectra in terms of hydrogen bonding is correct then pseudouridine-A_S (group II) forms a good hydrogen bond and A_F (group I) does not. Clearly neither the furanosyl (XVI) nor the pyranosyl (XX) structure for pseudouridine-A_S will permit a 5′-4-hydrogen bond. Inspection of molecular models indicates that *both anomers* of the furanosyl structure (XVI) can form hydrogen bonds from the 2′-oxygen to the 4-carbonyl group. A similar bond is possible in the β-pyranosyl structure, *but not in the* α

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[3] This is not the best ratio to show this effect, but more data are available at this ratio than at others.

TABLE II
PAPER CHROMATOGRAPHY OF PSEUDOURIDINE DERIVATIVES

Compound	Solvent[a] A	B	C
Pseudouridine-B	—	—	0.14
Pseudouridine-C	0.36	0.47	0.18
Acetone pseudouridine[b]	0.71	0.76	—
N-1-Cyanoethylpseudouridine[b]	0.41	0.57	0.26
N-1-Carboxyethylpseudouridine[b]	0.26	—	—
Acetone pseudouridine-5′-cyanoethylphosphate[b]	0.63[c]	0.64	—
Pseudouridine-5′-cyanoethylphosphate[b]	0.38[c]	0.47	—
Acetone pseudouridine-5′-phosphate[b]	—	0.60[c]	—
N-1-Cyanoethylpseudouridine-5′-phosphate	0.09	0.45	—
Pseudouridine-B-5′-phosphate	0.09	0.35	—
Pseudouridine-C-5′-phosphate	0.06	0.41	—
Pseudouridine-5′-phosphoramidate[b]	0.13	0.40	—
Pseudouridine-C-5′-diphosphate	0.05	0.37	—

[a] Solvent A, isopropyl alcohol–NH_4OH–H_2O (7:1:2); solvent B, isopropyl alcohol–1% $(NH_4)_2SO_4$ (2:1); solvent C, *n*-butyl alcohol–acetic acid–water (5:1:4). [b] Probably mixed isomers. [c] Whatman No. 4 paper.

anomer when the pyranosyl ring is in the C-1 conformation (XX, Fig. 10). This is the conformation one would predict to be most stable on the basis of nonbonded interactions (Reeves, 1950).[4] On the basis of this information we again favor the pyranosyl structures for A_S and A_F since it provides an explanation for the differences between the ultraviolet spectra of A_S and A_F in terms of a hydrogen bond. The furanosyl structures do not offer this clear-cut distinction.

From this discussion it is clear that the chemical behavior of pseudouridine can be rationalized. However, additional evidence for the structures of the A isomers and for the proposed mechanisms is needed. Work along these lines is in progress.

EXPERIMENTAL

Analytical Methods.—The methods used here have been described previously (Chambers *et al.*, 1960; Shapiro and Chambers, 1961). Pertinent R_F values for paper chromatography are given in Table II.

Ion-exchange chromatography was carried out on Dowex-1 ion-exchange resin, 200–400 mesh, 8% cross-linked unless stated otherwise. Gradient elution was carried out as described by Paar (1954). The notation 1 N $HCO_2H \rightarrow$ 2 N HCO_2H (500 ml) indicates that the concentration of eluent increased from 1 N to 2 N when 500 ml had passed onto the column. In all cases, the gradient was achieved by maintaining equal volumes of two solutions in two bottles. Thus, the gradient mentioned contained 250 ml of 1 N HCO_2H in the mixer and 250 ml of 2 N HCO_2H in the reservoir at the start. The ultraviolet-absorbing material was monitored with a Gilson absorption meter (253 mμ or 265 mμ). The recording was made on a Texas rectilinear recorder, and the curves shown in Figures 2, 5, and 6 are redrawn from these data. Since the recording system has not been calibrated for absorbance, arbitrary units have been used on the ordinates. Pseudouridine isomers were detected as described previously (Shapiro and Chambers, 1961).

Ultraviolet spectra were obtained with a Beckman DK 2 recording spectrophotometer and the curves were redrawn on a linear wavelength scale.

Isolation of Pseudouridine.—The following method is a modification of an unpublished procedure developed by W. E. Cohn.

Ten liters of normal human urine were collected without cooling or preservatives and stored overnight at 4°. About 9 l was decanted from any solid which settled out, and was passed through an 8.5 × 40–cm column of Amberlite IR-120-H$^+$.[5] The acidic effluent was passed through an 8.5 × 40–cm column of Amberlite IR-4B (free base form)[6] and the column was washed with water until the A_{260} fell below 1.0. The total volume of the effluent was about 15 l (TA_{260} = 80,000).[7] This fraction gave a negative test for inorganic phosphate and was neutral. Paper chromatography in solvent C gave a fluorescent spot near the origin, a light ultraviolet-absorbing spot corresponding to pseudouridine (R_F 0.23) and a darker ultraviolet spot just ahead of this.

Part of the combined effluent and washes from the second column (TA_{260} = 60,000) was made 0.005 M in $K_2B_4O_7$ and adjusted to *p*H 10 with concd NH_4OH. This solution was passed through a 4 × 15–cm column of Dowex-1-HCO_3^- at a flow rate of about 10 ml/min.[8] The column was then eluted with 0.005 M $K_2B_4O_7$ + 0.02 M NH_4HCO_3 (adjusted to *p*H 9 with ammonium hydroxide). After about 2 liters of the eluent had passed through the column, the A_{260} dropped somewhat and then a second large fraction requiring 3–4 liters of eluent was removed. Following this, a third small peak containing uracil, and usually some other unidentified compounds, emerged. The pseudouridine was then eluted with 0.1 M NH_4HCO_3 at a flow rate of about 15 ml/min. The pseudouridine can be identified by the large increase in A_{290} at *p*H 12 compared to that of the original effluent. The pseudouridine fraction was concentrated to about 250 ml. The ammonium bicarbonate that remained was removed by treating the solution with portions of Dowex-50W-H$^+$ until carbon dioxide evolution ceased. The resin was removed by filtration and washed well with water. The filtrate (TA_{260} = 12,000) was concentrated to dryness and the pseudouridine was crystallized from boiling 95% ethanol. The yield was 287 mg. This material had properties similar to those of an authentic synthetic sample (Shapiro and Chambers, 1961).

2′,3′-O-Isopropylidine Pseudouridine.—Dry pseudouridine (825 mg, 3.38 mmoles, dried over P_2O_5 *in vacuo* at 80° for 3 hours) was mixed with 2.2 g of di-*p*-nitrophenylphosphate (6.5 mmoles ∼ 6 equiv) and 4.2 ml of 2,2-dimethoxypropane (∼10 equiv) in 75 ml of acetone (dried over K_2CO_3 and distilled). The heterogeneous reaction mixture was stirred magnetically for 19 hours at room temperature in a stoppered flask.

The reaction mixture was cooled in ice and slowly

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[4] Cohn interprets his nuclear magnetic resonance data on A_s as follows: "The smaller spin coupling observed between C_2' and C_3' protons of A_s indicates an equatorial conformation for the C_3' H." This is consistent with the C-1 conformation.

[5] The resin (medium porosity, 20–50 mesh, capacity 5.0 meq/g) was used directly as supplied. The column was regenerated for further use with 20 liters of 2 N HCl.

[6] The resin (medium porosity, 20–50 mesh, capacity 5.5 meq/g) was used as supplied. Amberlite IR-45 has been used instead of IR-4B, but one batch of IR-45 absorbed all the pseudouridine and is, therefore, not recommended for this preparation. The column can be regenerated with 20 l of 4% NaOH.

[7] TA_{260} = absorbance at 260 mμ × ml, 1 cm light path.

[8] This resin was prepared by passing 5% sodium bicarbonate through a column of Dowex-1-chloride (8% cross-linked, 200–400 mesh) until the effluent gave a negative test for chloride ion using silver nitrate and nitric acid and then washing the column with water.

poured into a slurry of Dowex-1-HCO_3^- (80 ml) and ice cold water. Paper chromatography (solvent A) of an aliquot of this solution indicated the presence of about 4% pseudouridine. This was removed by adding 5 ml of Dowex-1-$B_4O_7^=$ and stirring the mixture for a few minutes.

The resins were removed by filtration and washed with 50% aqueous methanol. The combined filtrates were evaporated to dryness under reduced pressure and the residue was taken up in dry acetone. The product was crystallized by careful addition of hexane. The acetone pseudouridine was removed by filtration, washed with hexane, and dried in a desiccator over P_2O_5 and paraffin; 730 mg (76% yield); mp 208–210° (uncor); reported 233–234° (Michelson and Cohn, 1962). This material was used in the next step.

Ion-Exchange Fractionation of 2′,3′-O-Isopropylidene Pseudouridine.—Isopropylidene pseudouridine (36 mg) was dissolved in 10 ml of a 0.005 M $K_2B_4O_7$ + 0.02 M NH_4OH solution. Chromatography was carried out on a Dowex-1-Cl^- column (1 × 5 cm) using a double simultaneous linear gradient increasing in NH_4Cl and decreasing in $K_2B_4O_7$ and *p*H (Cohn, 1961): 0.005 M $K_2B_4O_7$ + 0.2 M NH_4OH → 0.02 M NH_4Cl (1 liter). The tubes corresponding to the peak (TA_{260} = 107) preceding the main fraction (TA_{260} = 814) were pooled and treated with charcoal (Barnebey-Chaney, 200 mg) for 20 minutes. The absorbed nucleoside was eluted with isopropyl alcohol–NH_4OH–H_2O (50:3:50; 30 ml, 89% recovery of ultraviolet-absorbing material). Paper chromatography revealed that this material was composed of two components: solvent A, R_F 0.62 (periodate +) and 0.70 (periodate −); and solvent B, R_F 0.76 (periodate +) and 0.80 (periodate −). The spectra of these components were similar to pseudouridine-C or A_B.

Pseudouridine-5′-Monophosphate.—Barium 2-cyanoethylphosphate (Tener, 1961) (3 g) was converted to its pyridium salt as described by Tener (1.5 × 9–cm column of Dowex-50-H^+). The stock solution (10 ml) contained 0.84 mmole phosphorus/ml. Six ml of stock solution (2 equiv) was added to 25 ml of dry pyridine (dried over CaH_2 and distilled) containing 709 mg (2.5 mmoles) of acetone uridine (dried over P_2O_5 *in vacuo* at 80° for several hours). Dicyclohexylcarbodiimide (3.09 g, 15 mmoles) was added and the reaction mixture was shaken mechanically for 3 days at room temperature. Paper chromatography (solvent A) showed the reaction was complete.

Water (5 ml) was added to the reaction mixture. After 1 hour the dicyclohexylurea was removed by filtration and washed well with water. The filtrate was evaporated to dryness under reduced pressure. The residue was taken up in a mixture of water and ether. The layers were separated and the ether layer was re-extracted twice with 10-ml portions of water. Chromatography of the combined aqueous layers (solvents A, B) revealed two major ultraviolet-absorbing phosphate-positive spots. The faster spot was periodate negative and was identified as 2′,3′-*O*-isopropylidene pseudouridine-5′-cyanoethylphosphate. The slower spot was periodate positive and was identified as pseudouridine-5′-cyanoethylphosphate. Loss of the isopropylidene group probably occurred during the evaporation of pyridine.

The aqueous layer was evaporated to dryness under reduced pressure and the residue was taken up in 30 ml of concentrated ammonium hydroxide. A small amount of insoluble solid (dicyclohexylurea) was removed by filtration and the filtrate was heated under a reflux condenser at 75° for 1 hour. Paper chromatography and paper electrophoresis indicated that the cyanoethyl groups had been removed completely giving two spots (solvent B) corresponding to pseudouridine-5′-phosphate and 2′,3′-*O*-isopropylidenepseudouridine-5′-phosphate.

The reaction mixture was evaporated to dryness under reduced pressure and the residue was taken up in 30 ml of 10% acetic acid. The *p*H was adjusted to 2.8 with glacial acetic acid and the mixture was heated at 80° for 1.5 hours. Chromatography (solvent A) and electrophoresis (*p*H 7 potassium phosphate buffer and *p*H 9.2 sodium borate buffer) indicated removal of the isopropylidene groups was complete. A trace ultraviolet-absorbing spot (unidentified) was detected in solvent A (R_F 0.19).

The reaction mixture was worked up by repeated evaporation of water under reduced pressure and the residue was taken up in 50.0 ml of water; TA_{260} = 16,930, 87% based on the TA_{260} of pseudouridine used.

This solution was fractionated by ion exchange as described by Tener (1961) using a 2 × 15–cm Dowex-1-formate column and a linear gradient elution, 1 N HCO_2H → 1.7 N HCO_2H (4 l). The results were similar to those obtained on a smaller run (Fig. 2). Fraction IV (TA_{260} = 1490) was evaporated to dryness at room temperature under reduced pressure and the residue was taken up in water. For further characterization see Characterization of Fraction IV (below).

Fraction V (TA_{260} = 10,900) was concentrated to 200 ml under reduced pressure and then lyophilized to dryness; 423 mg pseudouridine-5′-phosphoric acid (56% yield based on pseudouridine). This material gave a single spot in solvents A and B.

Anal. Calcd. for $C_9H_{13}N_2O_2P$ (324.19): P, 9.54. Found: P, 9.74.

The above material (409 mg) was dissolved in water (50 ml), neutralized to *p*H 7.0 with NH_4OH, and lyophilized to a white powder. Rechromatography of a portion of this ammonium salt (TA_{260} = 50) on a 0.8 × 15–cm Dowex-1-formate column using gradient elution 1 N HCO_2H → 1.6 N HCO_2H (600 ml) gave a single symmetrical peak corresponding to ψUMP-C.

Characterization of Fraction IV.—An aliquot of peak IV (see above) was fractionated by paper chromatography in solvent A. Two components, R_F 0.35 and R_F 0.45, were obtained. Both were periodate and phosphate positive. The spots were eluted with water and their A_{260} measured. The distribution was 38% and 62%, respectively. The solutions were evaporated to dryness in a vacuum desiccator and taken up separately in 0.1 ml of phosphate buffer *p*H 5.6. The solutions were treated with tobacco phosphatase for 2 days. The original R_F 0.35 material gave pseudouridine-B (characterized by paper chromatography, ultraviolet spectra, and ion exchange in the borate gradient system). Thus one component of fraction IV (the R_F 0.35 material) was the B isomer of pseudouridine-5′-phosphate (5-α-D-ribofuranosyluracil).

The R_F 0.45 material also gave a nucleoside with an R_F of 0.57 in solvent B. The nucleoside was isolated by paper chromatography in solvent A. The R_F 0.41 band was eluted with water. This material ran faster than pseudouridine in all 3 solvent systems (see Table II) and its ultraviolet spectrum (Fig. 3) showed no bathochromic shift at *p*H 12 compared to *p*H 7. The solution was evaporated to dryness in a vacuum desiccator, taken up in 20% NaOH, and heated at 100° for 15 minutes. The cooled solution was neutralized with Dowex-50W-H^+. The R_F of this material in solvent A was 0.26 (compared to 0.41 for the nucleoside *before* alkaline hydrolysis). Electrophoresis at *p*H 7 gave a minor spot corresponding to the nucleoside before alkaline hydrolysis and a major spot with approximately twice this mobility, indicating an increase of one negative charge. On this basis this product was assigned the structure of 1-*N*-carboxyethyl pseudouridine and the nucleoside from which it was derived by alkaline hydrolysis was then 1-*N*-cyanoethyl pseudouridine. Thus the R_F 0.45 (solvent B) component of fraction IV is 1-*N*-cyanoethyl pseudouridine-5′-phosphate.

Pseudouridine-5′-Phosphoramidate.—The ammonium salt of pseudouridine-5′-phosphate (C isomer, TA_{260} = 7700, ~ 1 mmole) was dissolved in a mixture of 7.5 ml of 2 N NH_4OH and 5 ml of formamide. Dicyclohexylcarbodiimide, 13.06 g, 15 mmoles, was dissolved in 20 ml of *tert*-butyl alcohol and added to the nucleotide solution. The homogeneous mixture was heated at 80° for 8 hours in a tightly stoppered flask and then allowed to stand overnight at room temperature. Paper chromatography (solvent A) indicated 12% unchanged starting material and 88% of the desired product.

The reaction mixture was worked up as described previously for uridine-5′-phosphoramidate (Chambers and Moffatt, 1958). Attempts to crystallize the product were unsuccessful. An amorphous solid was obtained by addition of acetone, 367 mg (59% yield based on ultraviolet measurements). This material was estimated to be about 75% pure. The mother liquors contained an additional 28% of the amidate.

The solid (above) was dissolved in 10 ml of water (TA_{260} = 4300) and fractionated on a column of Dowex-1-HCO_3^- (2.2 × 3 cm) using a linear gradient, 0.03 M NH_4HCO_3 → 0.1 M NH_4HCO_3 (2 l). The elution pattern is shown in Figure 5. ψUMP-NH_2 was isolated by evaporating the appropriate fractions (peak III) to a small volume under reduced pressure and lyophilizing the remaining solution to a white powder (170 mg). This material contained about 3% ψUMP. The formamide mother liquor (above) was worked up in a similar manner.[9] The total yield of ψUMP-NH_2 was about 63% based on ψUMP.[10]

The amidate was characterized as follows: 4 mg was incubated with crude *C. adamanteus* venom in Tris-HCl buffer (*p*H 8.8, 0.1 M) saturated with toluene at room temperature. After 6 days 82% of the material had been converted to pseudouridine C. The remaining material was not further degraded by additional enzyme. This material was isolated by paper chromatography (solvent A). It was hydrolyzed with 0.01 N HCl for 5 hours at room temperature and then with tobacco phosphatase in 0.1 M sodium acetate buffer (*p*H 5.6). The resulting nucleoside was identified as pseudouridine B by ion exchange (borate gradient) and its ultraviolet spectrum.

Peaks III and IV were not identified (Fig. 5). Peaks V and VI were characterized as ψUMP-B and C, respectively.

Pseudouridine-5′-Diphosphate.—Attempts to prepare ψUDP from the ammonium salt of ψUMP-NH_2 (above) under the usual conditions (Chambers *et al.*, 1960) gave unsatisfactory results (62% unchanged starting material). Therefore, ψUMP-NH_2 was converted to its 1,3-*N*-dicyclohexylguanidinium salt as follows: Four grams of dicyclohexylguanidine hydrochloride (sample from Lederle Laboratories) was suspended in 180 ml of water and enough methanol to bring the solid into solution was added. The solution was stirred with 25 ml of Dowex-1-OH^- resin and then poured through a 2 × 3-cm column of Dowex-1-OH^-. The effluent was concentrated to dryness under reduced pressure and the dicyclohexylguanidine (free base) was recrystallized twice from boiling aqueous methanol; 1.1 g, mp 180.5–182.5. ψUMP-NH_2 (ammonium salt, TA_{262} = 3230, 0.41 mmole based on ultraviolet) was dissolved in water (10 ml) and 91.2 mg (0.41 mmole) of dicyclohexylguanidine was added. Sufficient methanol to effect complete solution was added and the solution was evaporated to dryness (reduced pressure) several times. The residue was taken up in water and a small amount of insoluble solid was removed by filtration. The solution was lyophilized to a white powder, 228 mg. This material gave a negative test with Nessler's reagent and a positive test for dicyclohexylguanidine with 2 N HCl (Chambers and Moffatt, 1958).

This material (155 mg, 0.28 mmole) was dried at room temperature over P_2O_5 for 16 hours. Crystalline phosphoric acid (Fluka AG Buchs SG, Switzerland) was dried similarly; *o*-chlorophenol was redistilled. The reaction was set up as described previously (Chambers *et al.*, 1960) using *o*-chlorophenol (10 ml) as the solvent and anhydrous phosphoric acid (310 mg) instead of dioxane diphosphoric acid. The reaction was allowed to proceed for 3 hours at 0° and the mixture was worked up in the usual manner (Chambers *et al.*, 1960).

The reaction mixture (TA_{262} = 1740) was fractionated on a 15 × 10-cm column of Dowex-1-HCO_3^- resin. The elution pattern with NH_4HCO_3 is shown in Figure 6. The fractions corresponding to the double peak obtained with 0.18 M NH_4HCO_3 (72% of the ultraviolet-absorbing material put onto the column) were divided into three groups as shown in Figure 6. The middle cut was discarded and cuts IV_a and IV_c were worked up separately by concentration under reduced pressure and then by lyophilization.

The material in these cuts was identified by digesting a small sample with *C. adamenteus* venom in 0.3 ml of Tris-HCl buffer, 01. M, *p*H 8.8, and isolation of the nucleosides by paper chromatography (solvent A, Whatman No. 40). Hydrolysis of the material in cut IV_a was very slow and was incomplete after a week. The nucleoside produced was identified as pseudouridine-B by its spectra and by ion-exchange chromatography (borate buffer). The material in cut IV_c was hydrolyzed much more rapidly (complete in 24 hours) and the nucleoside was identified as the C isomer. The yield of β-ψUDP was 70 mg. Paper electrophoresis (sodium citrate buffer, *p*H 3.5) showed that it contained barely detectable trace of ψUMP.

Anal. Calcd. for $C_9H_{11}N_2O_{12}P_2 \cdot 3NH_4^+ \cdot 3H_2O$ (509.32): P, 12.20; total P/labile P, 2.00. Found: P, 12.16; total P/labile P, 2.06.

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[9] A similar pattern was obtained by chromatographing the mother liquor from the reaction mixture without isolating any solid prior to ion exchange. Thus the formamide solution containing the amidate may be chromatographed directly without precipitating the solid.

[10] The low yield was due to breakdown to ψUMP which occurred when Dowex-50-H^+ instead of lyophilization was used to remove the NH_4HCO_3. Direct fractionation of the formamide solution (see footnote 9) and lyophilization give the amidate in about 90% yield.

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Pyrimidines. XII. 1-Substituted 5-Azauracils and Related Compounds*

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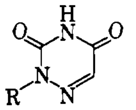
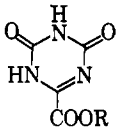
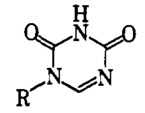
Base-catalyzed reactions of ethyl formate and 1-substituted biurets readily yielded 1-substituted 5-azauracils where the substituents were methyl, ethyl, cyclohexyl, benzyl, and furfuryl. The corresponding 5-azadithiouracils, where the substituents were methyl, cyclopentyl, cyclohexyl, and anisyl, were obtained from the reaction of thiocyanic acid and isocyanides. These compounds were synthesized in connection with the study of the relationship of 5-azauracils and 6-azauracils to the inhibition of pyrimidine biosynthesis.

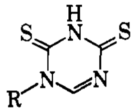
The carcinostatic activity of 6-azauridine (6-AzUR;[1] I. R = ribose) in certain types of leukemia, according to Handschumacher and co-workers (Jaffe *et al.,* 1957; Handschumacher and Pasternak, 1958; Handschumacher, 1960; Creasey and Handschumacher, 1961), is due to the blocking of the conversion of orotidylic acid (OMP) to uridylic acid in certain types of human acute leukemic cells by 6-AzUR and its corresponding nucleotide, 6-azauridine-5′-monophosphate (6-AzUMP), through inhibition of OMP decarboxylase in *de novo* pyrimidine synthesis (Fig. 1). Although 6-AzUR and 6-AzUMP are potent inhibitors of the enzyme orotidylic decarboxylase, the inhibition is a competitive one because their effectiveness in blocking this conversion is, at least to a considerable degree, overcome by the accumulation of orotidylic acid in the cells.


I

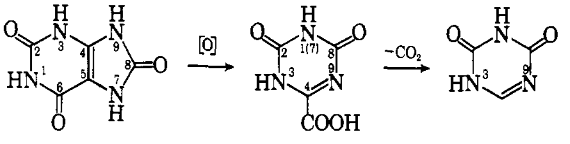
Subsequent to our investigation on the derivatives of orotic acid (Daves *et al.,* 1961), the 5-aza analog of orotic acid (2,4-dihydroxy-5-azapyrimidine-6-carboxylic acid, or oxonic acid), isolated as its potassium salt (II. R = K, Moore and Thomas, 1918; Hartman and Fellig, 1955) as well as its decarboxylated derivative 5-azauracil (or oxadin, III. R = H, Hartman and Fellig, 1955), were prepared in our laboratories. Compound II (R = K) has now been found (Handschumacher, 1962, 1963; A. D. Welch, personal communication) to interfere significantly with the conversion of orotate to orotydylic acid. Hence the oxonate inhibits the metabolic reversal of 6-AzUR (see Fig. 1), a fact which indicates that a combination of 6-AzUR (or 6-AzUMP) and oxonate can perhaps achieve significant carcinolytic effects. In order to understand the nature and effectiveness of oxonate in the role of pyrimidine biosynthesis, investigation of its closely related 1-substituted 5-azauracils and their thio analogs (IV) was therefore initiated as a logical extension of our studies in the orotic acid series.


II


III


IV

5-Azauracil (III. R = H) was obtained via 5-azaorotic acid (II. R = H) by the mild oxidation of uric acid (V). According to Brandenberger and Brandenberger (1954), the three nitrogen atoms in the triazine ring of II (R = H) originated from nitrogens 1 (or 7), 3, and 9 of V through one or more open-chain intermediates. Oxidation of 3- and 9-substituted uric acid under the


V II (R = H) III (R = H)

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[1] Abbreviations used in this paper: 6-AzUR, 6-azauridine; 6-AzUMP, 6-azauridine-5′-monophosphate; OMP, orotidylic acid.