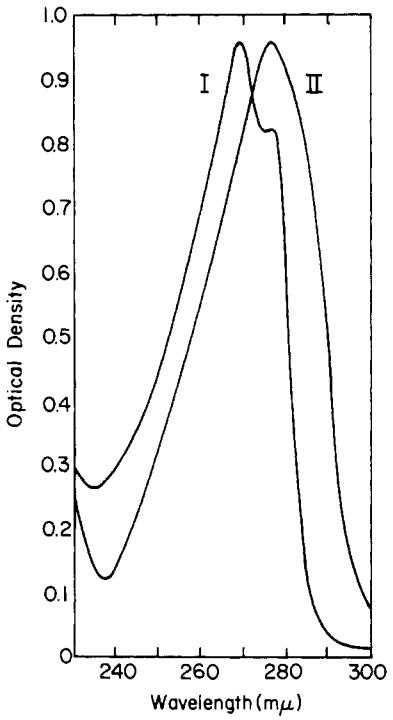

FIG. 8.—Ultraviolet-absorption spectra of the unknown compound Y_2 (Tables III and IV), I in 1 N alkali, and II in 1 N acid.

the determination of polynucleotide structure. However, further work on mixed s-RNA preparations would appear to be unprofitable and future efforts will be directed toward the isolation and study of a single s-RNA species.

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The Chemistry of Pseudouridine. III. The Structure of the A Isomers*

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Degradation of both pseudouridine A_F and A_S by periodate oxidation, borohydride reduction, and alkaline hydrolysis gave 5-(1′,2′-dihydroxyethyl)uracil. This establishes that the A isomers are anomeric forms of D-ribopyranosyluracil.

When pseudouridine-C is heated in either acid or alkali three new isomers, designated A_F, A_S, and B, are formed (Cohn, 1960; Chambers *et al.*, 1963). The structure of pseudouridine-C has been unequivocally established as 5-β-D-ribofuranosyluracil (Michelson and Cohn, 1962) and there is little doubt that pseudouridine-B is the α anomer of C. However there are at least four structures which are consistent with the data accumulated on the A isomers (Cohn, 1960; Shapiro and Chambers, 1961; Chambers *et al.*, 1963). Thus the A isomers could be pyranosyl (I) or furanosyl (IV) anomers or a combination of these forms. This paper describes a simple degradation scheme which clearly dis-

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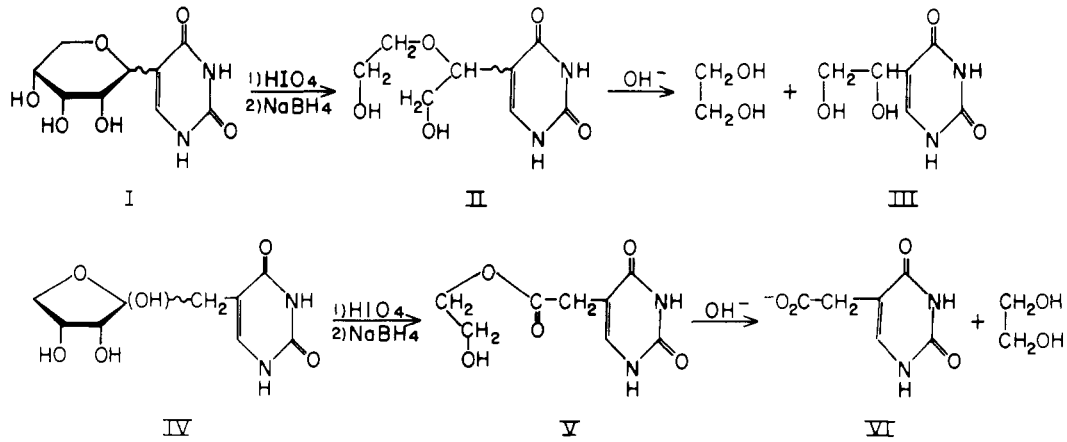

FIG. 1.—Degradation scheme for distinguishing between the two possible ring structures of the A isomers of pseudouridine.

tinguishes between these possibilities and provides evidence that the A isomers of pseudouridine are the α and β anomers of 5-D-ribopyranosyluracil (I).

The degradation scheme is shown in Figure 1. Actually, periodate oxidation suffices to distinguish compound I from IV, but, in practice, degradations of this type are often cleaner if one avoids working with the aldehydes. This is often accomplished by reducing them *in situ* to their corresponding alcohol derivatives (II and V in this case).

Neither the ether II nor the ester V are known compounds, but they should be converted to the simpler derivatives III and VI by alkaline hydrolysis. Compound V is an ester and its predicted alkaline lability needs no comment. Compound II is an ether and would not normally be alkali labile. However, on the basis of the mechanism postulated for the alkaline isomerization of pseudouridine (Chambers *et al.*, 1963), it could be predicted that II should undergo alkaline hydrolysis as shown in Figure 2.

The unsaturated intermediate VII (Fig. 2) might undergo several reactions. One of the more likely ones is addition of water to form 5-(1′,2′-dihydroxyethyl)-uracil (III). This glycol (III) derived from the pyranose I isomer would be easily differentiated from the acid (VI) derived from the furanosyl isomer (IV).

Pseudouridine-A_F was subjected to the first step in the degradation scheme. Two equivalents of periodate were taken up and after borohydride reduction a single ultraviolet-absorbing product was isolated in 76% yield by paper chromatography. This compound was relatively resistant to alkaline hydrolysis as would be expected of the ether II. However, after 2 hours at 100° in 1 N KOH, starting material and three new ultaviolet-absorbing compounds were detected by paper chromatography. One of these, the major *new* product, had properties consistent with 5-(1′,2′-dihydroxyethyl)-uracil (III).[1]

In order to confirm the structure of this alkaline hydrolysis product it was oxidized with periodate. A single ultraviolet-absorbing product was isolated by paper chromatography. This product was characterized as 5-formyluracil by its ultraviolet spectrum (Cline *et al.*, 1959). Reduction of the 5-formyluracil with $NaBH_4$ gave a product which was indistinguishable chromatographically and spectrally from an authentic sample of 5-hydroxymethyluracil.

These data combined with previous information (Cohn, 1960; Shapiro and Chambers, 1961; Chambers

[1] This compound contains an asymmetric carbon atom. Because of the nature of the hydrolysis the isolated product was undoubtedly racemic.

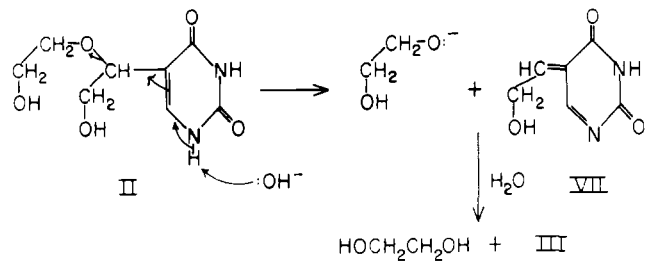

FIG. 2.—Postulated mechanism for the hydrolysis of II.

et al., 1963) conclusively establish the structure of pseudouridine A_F as 5-D-ribopyranosyluracil. A similar series of reactions establishes the same structure for pseudouridine-A_S (see under Experimental). Thus A_F and A_F must be α and β anomers. As discussed previously (Chambers *et al.*, 1963, Shapiro and Chambers, 1961), pseudouridine-A_F is probably 5-α-D-ribopyranosyluracil and pseudouridine-A_S is probably 5-β-D-ribopyranosyluracil. Further evidence on this remaining structural point will be reported separately.

EXPERIMENTAL

Analytical Methods.—Periodate uptake was measured spectrophotometrically (Dixon and Lipkin, 1954) using a Gilford Model 2000 multiple sample absorbance recorder and titrimetrically by the Müller-Friedberger method (Guthrie, 1962).

Paper chromatography was carried out on Whatman No. 40 paper by the descending method. The appropriate R_F values are given in Table I.

Ion-exchange chromatography and other procedures were carried out as described previously (Shapiro and Chambers, 1961; Chambers *et al.*, 1963).

TABLE I
R_F VALUES FROM PAPER CHROMATOGRAPHY

Compound	Solvent[a] I	II	III
Pseudouridine A_F	0.40	—	0.52
Pseudouridine A_S	0.33	—	0.42
Diglycol derivative (II) of A_F	0.59	0.77	—
Diglycol derivative (II) of A_S	0.55	—	—
5-(1′,2′-Dihydroxyethyl)uracil	0.46	—	—
5-Formyluracil	0.60	0.65	—
5-Hydroxymethyluracil	0.55	0.24	—

[a] Solvent I, isopropyl alcohol–NH_4OH–H_2O (7:1:2); solvent II, *n*-butyl alcohol–H_2O (86:14); solvent III, ammonium isobutyrate buffer (0.5 N NH_4OH–isobutyric acid, 6:10).

Preparation of the A Isomers of Pseudouridine.—Twenty mg of pseudouridine-C (Chambers *et al.*, 1963) was dissolved in 1 ml of 1 N HCl and heated at 100° for 1 hour. The reaction mixture was cooled and concentrated to about 0.2 ml by blowing a stream of N_2 over the solution. The entire mixture was streaked on Whatman No. 40 paper and chromatographed in solvent III. Pseudouridine B, C, and A_S ran together and A_F ran just ahead of them. The A_F band was eluted with water. Pseudouridine-A_S was isolated by a combination of ion-exchange and paper chromatography as described by Cohn (1960). Both these isomers were chromatographically and spectrally pure.

Degradation of Pseudouridine A_F.—The solution containing pseudouridine A_F (~10 μmoles, described above) was concentrated to dryness in a stream of N_2 and the residue was taken up in 0.6 ml of 0.1 M $NaIO_4$. The reaction tube was stoppered and allowed to stand in the dark at room temperature. Aliquots (25 μl) were removed periodically and the periodate concentration was determined by titration. The reaction was complete after 3 hours (periodate uptake = 2.4 moles/mole nucleoside). The excess periodate was destroyed by adding 3 μl (60 μmoles) of ethylene glycol and allowing the mixture to stand for 1 hour. Then 11 mg (300 μmoles) of $NaBH_4$ was added. After 3 hours at room temperature the reaction mixture was concentrated to 0.2 ml and fractionated by paper chromatography in solvent I. Two ultraviolet-absorbing bands were detected. One of these (R_F 0.68) was shown to be iodide ion. The other band (R_F 0.59) was eluted with water (76% based on pseudouridine-A_F).[2] This solution was evaporated to dryness and the residue was taken up in 0.5 ml of 1 N KOH. The mixture was heated for 2 hours at 100°, cooled, and neutralized with Amberlite IRC-50-H^+ ion-exchange resin (2 ml of wet resin). The resin was removed by filtration and washed with water (6 ml). The filtrate was evaporated to 0.2 ml and fractionated by paper chromatography in solvent I. Four ultraviolet-absorbing bands were detected: starting material (R_F 0.55), unidentified products R_F 0.29 and 0.73), and 5-(1′,2′-dihydroxy)uracil (R_F 0.46, see below for characterization, 17% yield).[2]

Degradation of Pseudouridine-A_S.—Pseudouridine-A_S (16 μmoles) was oxidized as described above for A_F. The reaction was complete in 2.5 hours. The procedure was modified as follows and is based on the method of Khym and Cohn (1960). The reaction mixture was poured onto a Dowex-1-acetate ion-exchange column (0.5 × 3.5 cm). The column was washed with 3 ml of 0.02 M acetic acid.

The combined effluent was concentrated to 2 ml and 16 mg (420 μmoles) of $NaBH_4$ was added. The mixture was allowed to stand overnight at room temperature. Then it was neutralized with Dowex-50-H^+ ion-exchange resin in the usual manner and the product (60% yield)[2] was obtained by evaporation of the filtrate. The product contained a trace of 5-(1′,2′-dihydroxyethyl)uracil presumably produced by the alkaline conditions which obtained during $NaBH_4$ reduction.

The crude product was hydrolyzed for 1 hour at 100° in 1 N KOH and the mixture was fractionated directly by paper chromatography in solvent I. Besides starting material, an unidentified side product (R_F 0.65) and 5-(1′,2′-dihydroxy)uracil (R_F 0.35, 13% yield) were detected.[3]

Characterization of 5-(1′,2′-Dihydroxyethyl)uracil.—The product derived from the degradation of pseudouridine A_F and A_S (1 μmole[2] from each in 0.1 ml of water) was oxidized with excess $NaIO_4$ (2–3 μmoles) in the usual manner.[4] The excess periodate was destroyed with ethylene glycol (2–8 μmoles) and the mixtures were fractionated by paper chromatography in solvent I. An aliquot from the reaction mixtures showed the same product (judged by paper chromatography in solvents I and II and by ultraviolet spectrophotometry) was formed. This product was characterized as 5-formyluracil by its ultraviolet spectrum (Cline *et al.*, 1959).

Twenty minutes after addition of the ethylene glycol (above), 2 mg (53 μmoles) of $NaBH_4$ was added to each oxidation mixture. After 16 hours the mixtures were examined by paper chromatography. The product from both reactions was identical with an authentic sample of 5-hydroxymethyluracil (Mann Research Laboratories, New York) both chromatographically (3 solvents) and spectrally (ultraviolet).

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[2] This calculation is based on the assumption that the $\epsilon_{max}^{pH\ 7}$ of the oxidation product of pseudouridine is the same as pseudouridine-C.

[3] The R_F values were slightly different from those obtained with A_F. This was due to the high concentration of inorganic ions.

[4] In separate quantitative runs the product from A_S took up 0.72 mole of periodate per mole of nucleoside; A_F took up 0.67 mole/mole. The low values are probably due to deviation of the assumed ϵ_{max} for the pyrimidine derivative from the true value (see footnote 2).