STUDIES ON AN ISOMER OF URIDINE ISOLATED FROM RIBONUCLEIC ACIDS

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SUMMARY

1. The nucleoside component of the new unknown nucleotide discovered by DAVIS AND ALLEN has been isolated from yeast. A large scale preparation of this unknown nucleoside from commercial uridine purchased from the Schwarz Laboratories, Inc., has been described.

2. The unknown nucleoside is found to have a composition identical to that of uridine.

3. The ultraviolet absorption spectra of the unknown nucleoside at several pH values are presented, and found to show close resemblance to that of the unknown nucleotide.

4. The oxidation of uridine and of the unknown nucleoside has been found to require 0.98 and 0.99 mole of periodate respectively per mole of nucleoside which indicates that the carbohydrate component of the unknown nucleoside has a furanose structure.

5. Periodate oxidation followed by treatment with bromine results in the formation of large quantities of uracil and 5-bromouracil from uridine and from the unknown nucleoside. These findings indicate that the pyrimidine component of the unknown nucleoside is uracil. Earlier work has shown the carbohydrate component of the unknown nucleoside to be ribose. Thus the unknown nucleoside is a uracil-ribofuranoside and is an isomer of uridine.

6. The absorption spectra of the unknown nucleoside at pH 7 and 12 resembles those of 5-hydroxymethyl uracil, which may indicate that the carbohydrate component is attached to the pyrimidine component through the 5 position.

INTRODUCTION

With the improvement of chromatographic methods and of isolation procedures as applied to ribonucleic acids in recent years, certain new components in ribonucleic acids have been found[1-7]. By the use of two-dimensional paper chromatograms, DAVIS AND ALLEN[1] discovered a new unknown nucleotide in the hydrolysates of ribonucleic acids from yeast. This unknown nucleotide has also been found in pancreas[2], wheat germ ribonucleic acids, and the microsome fraction of the blood cells*.

* Unpublished observation.

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The isolation and certain properties of this unknown nucleotide have been described[1]. The most notable difference in the properties of the unknown nucleotide from that of other naturally-occurring nucleotides is a pronounced bathochromic shift in its ultraviolet absorption spectrum at alkaline pH values. Since the unknown nucleotide is liberated by ribonuclease to the same extent as are the pyrimidine nucleotides, the non-purine nature of its nitrogenous component has been suggested. The carbohydrate component was found to be ribose.

The present communication reports the isolation, purification and characterization of the nucleoside component of this unknown nucleotide. Elementary analysis shows that the unknown nucleoside has a composition identical to that of uridine. Furthermore, evidence is presented which indicates that the nitrogenous component of the unknown nucleoside is uracil and the pentose component has a furanose structure. Since earlier work[1] has shown the pentose to be ribose, the unknown nucleoside, as a uracil-ribofuranoside, is an isomer of uridine. The linkage between the pyrimidine and the carbohydrate components appears to be the only possible difference between uridine and the unknown nucleoside.

EXPERIMENTAL AND RESULTS

Materials. Baker's yeast (*Saccharomyces cerevisiae*) was supplied directly from the filter press by the Consumers yeast Company, Oakland, California. Uridine was purchased from the Schwarz Laboratories, Inc. (Lots UN5303 and UN5703), crystalline ribonuclease from the Worthington Biochemical Corp. (Lot 556), bovine intestinal phosphatase from Pentex, Inc. (Lot B 3103), 5-bromouracil and 5-hydroxymethyluracil from California Foundation for Biochemical Research (Lots 2041 and 3950), and 4-methyluracil from Nutritional Biochemical Corporation (Lot 9483).

The paper chromatographic and paper electrophoretic procedures were carried out according to the methods of CRESTFIELD AND ALLEN[8,9].

Preparation of the nucleoside component of the unknown nucleoside from yeast. Ribonucleic acids from yeast were extracted with sodium dodecylsulfate by the method to CRESTFIELD, SMITH AND ALLEN[10]. The first ethanol precipitate of the dodecylsulfate extract of 75 g baker's yeast was washed three times with 75 ml portions of 67, 67 and 80 % cold ethanol respectively. The precipitate was dissolved in 25 ml cold freshly boiled distilled water. Five mg crystalline ribonuclease was added to the crude ribonucleic acids preparation, and the reaction mixture was allowed to stand at room temperature for 8 h. The amount of pyrimidine nucleotides was estimated according to CRESTFIELD *et al.*[10] to be 125–175 mg. Fifteen mg of bovine intestinal phosphatase (10 % of the substrate), dissolved in 8 ml of a 0.2 *M* Tris buffer at pH 9 were added to the hydrolysate. After 4 h standing at room temperature, the reaction mixture was subjected directly to paper chromatography as a narrow zone on six sheets of Whatman No. 1 paper (18 × 9 in.). The sheets were developed in isopropanol–acetic acid–water solvent (60:30:10, v/v/v)[11] ascending for 24 h. After each flow of the solvent the sheets were dried and resubmitted to the same solvent for a total of ten 24-h periods. The ultraviolet-absorbing zone which contained the pyrimidine nucleosides was cut from the paper, eluted and submitted to ascending paper chromatography in isobutyric acid–0.5 *M* ammonia solvent (10:6, v/v)[12], whereby cytidine was separated from the combined zone of uridine and the unknown nucleoside. The latter area was

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cut from the paper, eluted and submitted to paper chromatography in butanol–water (84:16, v/v)[13]. Uridine moved out rapidly in this solvent system whereas the unknown nucleoside had an R_F value approximately half that of uridine.

Large scale preparation of the unknown nucleoside. Commercial uridine purchased from the Schwarz Laboratories, Inc. was found to contain 0.6 % of the unknown nucleoside. The unknown nucleoside from this source was found to be the same as that prepared from baker's yeast. A comparison of the spectral data of the unknown nucleoside prepared from commercial uridine and from yeast is given in Table I.

TABLE I

SPECTRAL DATA ON UNKNOWN NUCLEOSIDE

Source of material	*pH*	λ_{max} mμ	ε_{max}* × 10^3	λ_{min} mμ	ε_{min}* × 10^3	ε_{260}*	*Ratio* 250/260	280/260	290/260	300/260
Commercial uridine	2	263	7.5	232	2.0	7.4	0.74	0.42	0.06	0
	12	286	7.3	245	1.7	3.2	0.60	2.11	2.22	1.21
Yeast	2	263	—	234	—	—	0.74	0.43	0.07	0
	12	286	—	245	—	—	0.64	2.09	2.16	1.22

* Calculated on the assumption that the molecular weight of the unknown nucleoside is identical to that of uridine.

Two g of uridine dissolved in 2 ml of distilled water were applied as a narrow zone on 12 sheets of Whatman No. 1 paper (18 × 9 in.). The sheets were submitted to the butanol–water solvent ascending for a total of fourteen 24-h periods. By this time uridine was concentrated as approximately a 3 in.-wide zone which was located about 10 in. above the site of application. The unknown nucleoside followed uridine immediately as a 1 in.-wide zone. The zone which contained the unknown nucleoside was cut from the 12 sheets of paper and extracted with two 150 ml portions of distilled water. The extracts were combined, and the volume was reduced under vacuum. For further purification, the concentrated unknown nucleoside solution was resubmitted to paper chromatography by the use of butanol–water solvent and to paper electrophoresis with ammonium formate buffer at pH 9.2 and ionic strength 0.2. The preparation at this time appeared slightly yellow. The yellow color was completely removed by washing the preparation with ice cold water. Small amounts of the unknown nucleoside were recovered from the combined washings by the use of paper chromatography. The yield from each 2 g portion of uridine ranged from 10 to 14 mg. In addition to the unknown nucleoside, commercial uridine was found to contain numerous minor components which were resolved into seven utraviolet-absorbing zones in this chromatographic system. The $O.D._{260}$ of the materials which were contained in the various zones varied from 0.01 to 0.1 % of the total $O.D._{260}$.

Elementary analysis. The elementary composition of the unknown nucleoside was found to be identical to that of uridine.

	C	*H*	*N*	*O*
Uridine $C_{19}H_{12}N_2O_6$, calculated	44.26	4.95	11.47	39.22
The unknown nucleoside, found	44.70	4.90	11.61	38.82

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The melting point of the unknown nucleoside, 209.5°–210.5°, however, is considerably higher than that of uridine, 165°*. The optical rotation of the unknown nucleoside, $[\alpha]_D$, was found to be —3.0° and that of uridine, +8.5°, measured in a 1 % solution in water*,**.

Spectral data

The ultraviolet absorption spectra of the unknown nucleoside, measured at several pH values are shown in Fig. 1. The pronounced bathochromic shift which was noted with the unknown nucleotide as reported previously is also observed with the nucleoside component. Other spectral data on the unknown nucleoside also show close resemblance to that of the unknown nucleotide. The unknown nucleoside contains a dissociation group with a pK of 9.2 as determined by the spectrophotometric method of EDWARDS[14] and which may be compared to a value of 9.6 which has been published for the unknown nucleotide[1].

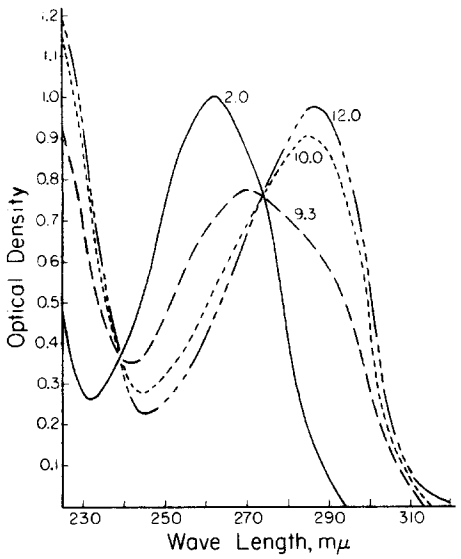

Fig. 1. Absorption spectra of the unknown nucleoside at pH values indicated. The curves for pH 1 5.4, and 7.3, not shown, are identical with that for pH 2.0.

In 0.1 N, 1 N and 2 N solutions of sodium hydroxide, the absorption of the unknown nucleoside at 280 mμ (ε_{280}) was found to be 82, 77 and 11 % of that at pH 12 respectively. Since the wave length of the maximum absorption (λ_{max}) was not altered, it seems unlikely that the lowered values of ε_{280} in concentrated solutions of sodium hydroxide were due to the presence of an additional dissociation group. When the pH of the solutions was brought to 12 with concentrated hydrochloric acid, the values of ε_{280} were found to be equal to those at pH 12. Thus no destruction of the unknown nucleoside occurred. Since the nitrogenous component of the unknown nucleoside will be shown later to be uracil, it is possible that the lowered values of ε_{280} are due to the addition of a molecule of water to the 4–5 double bond in concentrated

* Thanks are due Dr. A. M. CRESTFIELD for assistance in measuring the melting points.
** Thanks are due to Dr. C. E. BALLOU for assistance in measuring the optical rotations.

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solutions of sodium hydroxide. Similar reversible reactions were found when derivatives of uracil were exposed to ultraviolet irradiation[15].

Periodate oxidation

The consumption of periodate by the vicinal glycol groups of the unknown nucleoside and uridine was followed spectrophotometrically by the method of DIXON AND LIPKIN[16]. The unknown nucleoside consumes 0.99 mole of meta sodium periodate per mole of nucleoside as compared with 0.98 mole per mole of uridine.

Periodate oxidation of uridine and the unknown nucleoside followed by acid or alkali treatment

The periodate oxidation product of uridine was incubated with 1, 2 and 4 *N* solutions of ammonium hydroxide at room temperature. Aliquots of the reaction mixture were taken at time intervals which varied from 1 to 150 h and were subjected to paper chromatography in butanol–water solvent ascending for 8 h. After the 7th h of incubation in the alkali, trace amounts of uracil appeared on the paper and the characteristic trailing of the dialdehyde on paper chromatograms began to fade. After 150 h of incubation a single ultraviolet-absorbing substance which moved out of the site of application on the paper was found to be uracil. Other alkaline reagents tested for this reaction included ammonium formate buffer and glycine buffer at pH 10. 4 *N* ammonium hydroxide gave a better yield of uracil than any of the other reagents tested. This alkali-catalyzed reaction was employed by WHITFELD[17] for the production of cytosine from the periodate oxidation products of cytidine.

Similar experiments were carried out with the unknown nucleoside; the periodate oxidation product of the unknown nucleoside was incubated with 4 *N* ammonium hydroxide at room temperature. Samples of the reaction mixture were taken at time intervals which varied from ½ to 115 h and were subjected to paper chromatography. In all cases light blue fluorescent materials appeared as streaks on the paper. No ultraviolet-absorbing material was detectable.

Periodate oxidation products of uridine and of the unknown nucleoside were also treated with 1 *N* hydrochloric acid at room temperature. Samples were taken at 5 min, ½, 1 and 2 h intervals and were subjected to paper chromatography in butanol–water solvent. The dialdehyde derivative of uridine formed an ultraviolet-absorbing compound on the paper which was found to be uracil. The dialdehyde derivative of the unknown nucleoside, however, again failed to form any distinct ultraviolet-absorbing compound on the paper. The acid-catalyzed reaction was employed by LYTHGOE AND TODD[18] for the production of theophylline from the periodate oxidation product of theophylline-7-*d*-glucoside.

Acid hydrolysis and bromination of the unknown nucleosides

1-mg samples of uridine and of the unknown nucleoside were dissolved in separate test tubes with 15 µl of 12 *N* perchloric acid[19]. The tubes were heated in a boiling water bath and samples were taken at 15-, 30-, 60- and 120-min intervals. The samples were diluted with 10 portions of distilled water and subjected to paper chromatography in butanol–water solvent. In the case of uridine a distinct ultraviolet-absorbing spot corresponding to uracil was found as the only product on the paper chromatograms. In the case of the unknown nucleoside, however, very light ultraviolet-ab-

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sorbing streaks appeared around the region of uracil. Thus, most, if not all, of the unknown nucleoside was destroyed under these conditions. Consequently, milder conditions were used. 380 μg of the unknown nucleoside in 0.2 ml of 1 N sulfuric acid was heated for 1 h in a boiling water bath[20]. The reaction mixture was then subjected to ascending paper chromatography in butanol–water solvent for 12 h. The paper was dried and resubmitted to the same solvent for six additional times. Two major zones and one minor zone of ultraviolet-absorbing materials appeared on the paper. The leading major zone (zone 1) had an R_F value exactly the same as that of the unknown nucleoside. This was followed immediately by the minor zone (zone 2) which, in turn, was followed immediately by the second major zone (zone 3). For further purification, the various zones were cut from the paper, extracted with distilled water, and resubmitted to paper chromatography in butanol–water solvent. The spectral ratios of the various zones at pH 2 and 12 were measured. As shown in Table II, the acid conversion products of the unknown nucleosides have similar, but not identical, spectral ratios at pH 2 and 12. These ratios, furthermore, are different from those corresponding to the unknown nucleoside.

TABLE II

SPECTRAL RATIOS OF THE ACID CONVERSION AND BROMINATION PRODUCTS OF THE UNKNOWN NUCLEOSIDE

Treatment	*Zone*	*pH 2*			*pH 12*		
		250/260	*280/260*	*290/260*	*250/260*	*280/260*	*290/260*
Acid	1	0.71	0.42	0.05	0.63	1.78	1.74
Bromine	1	0.73	0.43	0.06	0.63	1.84	1.83
Acid	2	0.73	0.50	0.07	0.65	1.53	1.56
Bromine	2	0.69	0.48	0.07	0.65	1.52	1.52
Acid	3	0.76	0.35	0.03	0.55	2.51	2.59
Bromine	3	0.76	0.37	0.03	0.55	2.39	2.46

Bromination of the unknown nucleoside was carried out according to the method of LEVENE AND LA FORGE[21]. 380 μg of the nucleoside in aqueous solution were treated with excess bromine. The excess bromine was evaporated by the use of a stream of nitrogen. The ultraviolet absorption spectrum of the brominated solution was measured. The brominated unknown nucleoside showed only end-absorption in the ultraviolet. This end-absorption in the ultraviolet is characteristic of a pyrimidine derivative with a saturated bond between the 4 and 5 position of the pyrimidine ring[15]. The brominated solution of the unknown nucleoside was dried and the residue was dissolved in absolute ethanol and boiled in a water bath for 1 h. The reaction mixture was then subjected to paper chromatography in butanol–water solvent. Three zones similar to the acid conversion products appeared on the paper. Each zone had an identical R_F in this chromatographic system with the corresponding zone of the acid conversion product. The spectral ratios of these zones at pH 2 and 12, as shown in Table II, are identical to those corresponding to the acid conversion products. It seems very likely, therefore, that the corresponding zones of the unknown nucleoside treated with 1 N sulfuric acid and with bromine are identical compounds. It is of

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interest that the unknown nucleoside which was brominated by the addition of bromine as shown by the loss of its ultraviolet absorpton was debrominated upon boiling in alcohol. LEVENE AND BASS[22] have considered the bromination of uridine and postulated that 4-hydroxy-5,5-dibromohydrouridine was the intermediate. Upon boiling in alchol, the 4–5 bond became desaturated and resulted in the formation of 5-bromouridine.

Periodate oxidation of uridine and the unknown nucleoside followed by bromination

For the isolation of the pyrimidine component from the corresponding pyrimidine nucleoside several approaches are available: (a) oxidation of the carbohydrate component with hot 12 *N* perchloric acid[19], (b) oxidation of the carbohydrate component with periodate followed by treatment with alkali[17], and (c) mild hydrolytic procedures after saturation of the 4,5-double bond of the pyrimidine ring[29]. As described previously, approaches (a) and (b) resulted in the formation of uracil from uridine but failed to give any detectable product which absorbs ultraviolet from the unknown nucleoside. Similarly bromination and also hydrogenation as reported by COHN[7] (approach (c)) failed to give any clue as to the identification of the pyrimidine component of the unknown nucleoside. Thus it appears that the logical step to be followed is a combination of the above approaches.

Pilot experiments indicated that after the periodate oxidation of uridine, bromination of the dialdehyde would give rise to uracil and 5-bromouracil. Since iodate produced from periodate during the oxidation was found to interfere with the subsequent bromination of the dialdehyde derivative, it must be removed from the reaction mixture. The removal of iodate was accomplished by paper electrophoresis. The amount of periodate used was slightly less than that of uridine to avoid the presence of residual periodate which was more difficult to remove from the reaction mixture than its reduction product, iodate. 5-Bromouracil and uracil were also obtained when the unknown nucleoside was treated similarly. The experiments were carried out as described in the following paragraph.

11.2 μmoles of sodium metaperiodate was added to 12.4 μmole of the unknown nucleoside. The reaction mixture was then immediately subjected to paper electrophoresis with ammonium formate buffer at pH 9.2 and ionic strength 0.2. After 1 h electrophoresis (18 V/mm) the periodate reduction product, iodate, bearing a negative charge at this pH, moved toward the cathode, whereas the dialdehyde formed from the nucleoside by periodate oxidation remained at the site of application. The dialdehyde was eluted from the paper, dried with a stream of nitrogen and dissolved in 500 μl distilled water. Excess bromine (5 μl) was added and the solution was allowed to stand for 15 min. The reaction mixture was then dried and heated in absolute ethanol in a boiling water bath for 2 h. The reaction mixture was applied to one sheet of Whatman No. 1 paper (14 × 9 in.). The paper was developed in butanol–water solvent ascending for 12 h. Resubmission of the paper to the same solvent for several times was necessary in order to separate the various ultraviolet-absorbing zones from each other. Areas corresponding to 5-bromouracil and uracil were cut from the paper, eluted and resubmitted to paper chromatography in the same system to free them from trace contamination of ultraviolet-absorbing material. The products were identified as 5-bromouracil and uracil by comparison with the known reference compounds with respect to spectra at pH 2, 7 and 12, as well as R_F values in various

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TABLE III

COMPARISON OF SPECTRAL DATA BETWEEN 5-BROMOURACIL AND 5-BROMOURACIL DERIVED FROM THE UNKNOWN NUCLEOSIDE

Source of material	*pH*	λ_{max} mμ	λ_{min} mμ	*Ratio*							
				240/260	*250/260*	*270/260*	*280/260*	*290/260*	*300/260*	*310/260*	*320/260*
Reference	1	276	241	0.33	0.52	1.47	1.50	0.95	0.28	—	—
	2	276	241	0.32	0.51	1.48	1.51	0.95	0.27	—	—
	12	298	253	1.70	0.84	1.65	2.51	3.34	3.59	2.53	0.73
Unknown nucleoside	1	276	241	0.33	0.52	1.47	1.50	0.95	0.28	—	—
	2	276	241	0.32	0.52	1.47	1.51	0.95	0.28		
	12	298	253	1.73	0.86	1.65	2.54	3.39	3.54	2.55	0.74

TABLE IV

COMPARISON OF SPECTRAL DATA BETWEEN URACIL AND URACIL DERIVED FROM THE UNKNOWN NUCLEOSIDE

Source of material	*pH*	λ_{max} mμ	λ_{min} mμ	*Ratio*			
				250/260	*270/260*	*280/260*	*290/260*
Reference	2	259	—	0.84	0.67	0.17	—
	12	284	241	0.71	1.21	1.40	1.26
Unknown nucleoside	2	259	—	0.85	0.68	0.17	—
	12	284	241	0.70	1.20	1.37	1.24

paper chromatographic systems and relative mobilities in various electrophoretic systems described in the preceding paragraphs. Comparison of spectral data between reference 5-bromouracil and 5-bromouracil derived from the unknown nucleoside is given in Table III. Similar comparisons for uracil are given in Table IV. The molar extinction coefficient reported by DUNN AND SMITH[23] was used to estimate quantitatively the amount of 5-bromouracil which was produced. The molar extinction coefficient provided by JOHNSON[24] was employed to determine the amount of uracil which was produced. The amount of the unknown nucleoside which was recovered as 5-bromouracil was found to be 7.2 % and that recovered as uracil was 8.1 %. Since 5-bromouracil failed to give rise to any detectable uracil when boiled in absolute ethanol, uracil obtained from the unknown nucleoside could not be the debrominated product of 5-bromouracil. The absorption spectra of 5-bromouracil at pH 2 and 12 are shown in Fig. 2.

The bromination products of the dialdehyde derivative of uridine contained three ultraviolet-absorbing zones which corresponded to 5-bromouracil, uracil, and some residual uridine. In the case of the unknown nucleoside, there were several other zones in addition to that of 5-bromouracil and uracil. These additional zones are probably those which contain the pyrimidine and some form of the oxidized residue of the carbohydrate. The differences which are observed between the bromination products of the dialdehyde of uridine and that of the unknown nucleoside can be explained by postulating a more stable bond between the pyrimidine ring and the oxidized residue of the carbohydrate in the case of the unknown nucleoside.

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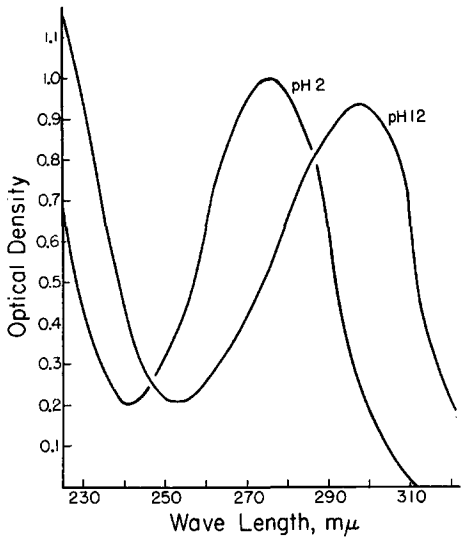

Fig. 2. Absorption spectra of 5-bromouracil at pH values indicated. The curve for pH 1, not shown, is identical with that for pH 2.

DISCUSSION

Elementary analysis of the unknown nucleoside has shown this compound to have a composition identical to that of uridine. Earlier work showed the carbohydrate component of the unknown nucleoside to be ribose[1]. Thus the nitrogenous component of the unknown nucleoside is either an isomer of uracil or uracil itself. It is concluded to be uracil from the following facts: (a) Uracil and 5-bromouracil are obtained from the unknown nucleoside in amounts corresponding to 8.1 and 7.2% of the starting material respectively. (b) Under the present experimental conditions there are no known chemical reactions which would lead to the formation of uracil from a pyrimidine derivative which is an isomer of uracil and yet different from uracil.

The amounts of sodium metaperiodate required for the oxidation of uridine and of the unknown nucleoside requires 0.98 and 0.99 mole periodate respectively per mole of the nucleoside. It is known that the oxidation of a pyranoside which has three adjacent carbon atoms carrying free hydroxyl groups requires 2 moles of periodate per mole of the compound. On the other hand, the oxidation of a furanoside which has two adjacent carbon atoms carrying free hydroxyl groups requires only 1 mole. Thus LEVENE AND TIPSON[25] found that the oxidation of adenosine and guanosine requires 1.04 and 1.00 mole periodate per mole of the nucleosides, respectively. Similarly, DIXON AND LIPKIN[16] reported that the oxidation of adenosine, guanosine, cytidine and uridine requires 0.98, 0.99, 1.07 and 1.00 mole periodate respectively. The finding that the oxidation of the unknown nucleoside requires 0.99 mole periodate per mole of the nucleoside strongly suggests that its carbohydrate component has a furanose structure. Earlier work has shown the carbohydrate to be ribose[1]. Thus, the unknown nucleoside is a uracil-ribofuranoside, and is an isomer of uridine.

Since both the pyrimidine and the carbohydrate components of the unknown

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nucleoside are the same as those of uridine, the only possible difference between these two nucleosides is the linkage between these components.

Incubation of the dialdehyde derivatives of uridine and of the unknown nucleoside at alkaline pH resulted in the formation of uracil from uridine but not from the unknown nucleoside. WHITFELD[17] has employed a similar procedure for the degradation of cytidine and found that free cytosine is formed. Since uracil and cytosine are obtained from the corresponding pyrimidine nucleosides with these procedures, it appears that the normal N–C linkage between the pyrimidine and the carbohydrate components is labile to these conditions. The fact that the linkage in the unknown nucleoside is more resistant to these conditions suggests that this linkage is not the normal N–C linkage. The other possible linkage then would involve a C–C bond.

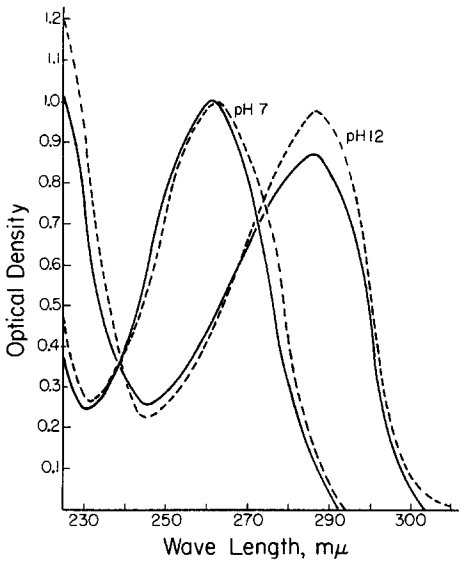

Fig. 3. Absorption spectra of the unknown nucleoside (dotted lines) as compared with 5-hydroxymethyluracil (straight lines) at pH values indicated.

Comparisons between the ultraviolet absorption spectra of various methylated derivatives of uracil and that of uridine and the unknown nucleoside were made. The spectra of 4-methyluracil and 5-hydroxymethyl uracil were measured at pH 7 and 12, and SHUGAR AND FOX[26] have described the spectra of 1-methyluracil, 3-methyluracil and thymine (5-methyluracil) at various pH. It was noted that there is a striking resemblance between the spectra of 3-methyluracil and uridine, and also between that of 5-hydroxymethyluracil and the unknown nucleoside. The spectra of 5-hydroxymethyluracil and the unknown nucleoside at pH 7 and 12 are shown in Fig. 3. The only major variation in the spectra of these two compounds is the ε_{max} at pH 12. In the case of 5-hydroxymethyluracil ε_{max} at pH 12 is 13% lower than that at pH 7, and in the unknown nucleoside it is 3% lower. Similar variations were also observed in the spectra of uridine and 3-methyluracil; their ε_{max} values at pH 12 are 16 and 29% respectively lower than the corresponding values at pH 7. The spectral data of 1-methyluracil, 4-methyluracil, and thymine, as shown in Table V, also resembles that of the unknown nucleoside. However the values of the ε_{max} of these three compounds

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at pH 12 are 47% higher, 32% lower and 32% lower respectively than the corresponding values at pH 7 and hence are very different from that of the unknown nucleoside. The close resemblance of the spectra of 5-hydroxymethyluracil and the unknown nucleoside suggests that in the unknown nucleoside the carbohydrate component is attached to the 5 position.

TABLE V

COMPARISON OF SPECTRAL DATA BETWEEN METHYLATED DERIVATIVES OF URACIL AND URIDINE AND THE UNKNOWN NUCLEOSIDE

	λ_{max} (mμ)		λ_{min} (mμ)		ε_{max} pH 7 × 10^{-3}	$\frac{\varepsilon_{max}\ pH\ 12}{\varepsilon_{max}\ pH\ 7}$
	pH 7	pH 12	pH 7	pH 12		
3-Methyluracil*	266	265	233	241	9.8	0.71
Uridine	262	262	230	236	10.1	0.87
The unknown nucleoside	263	286	232	245	7.5	0.97
5-Hydroxymethyluracil	261	286	231	246	—	0.87
1-Methyluracil*	259	283	230	243	7.3	1.47
4-Methyluracil	260	275	230	246	—	0.68
Thymine*	265	291	234	245	7.9	0.68

* SHUGAR AND FOX[26].

The above postulate can be used to interpret the production of uracil and 5-bromouracil from the unknown nucleoside. The reaction leading to the formation of uracil and 5-bromouracil may be considered as shown in Scheme I. Compound I is the postulated structure of the unknown nucleoside. Periodate oxidation of the unknown nucleoside results in the formation of the corresponding dialdehyde derivative (Compound II). The function of bromine is two fold: (a) the bromination of the pyrimidine ring (Reaction 2) and (b) the oxidation of the aldehyde group to the carboxylic acid (Reaction 2a)[27]. Thus bromination may eventually result in the formation of Compound V. In the bromination of uracil, 5,5-dibromo-4-hydroxyhydrouracil was considered to be the initial product[28]. During the subsequent boiling in ethanol, one of the two bromine atoms and the hydroxyl group were eliminated from the 5 and 4 positions of the ring respectively, resulting in the formation of 5-bromouracil. With the periodate oxidation product of the unknown nucleoside, however, the presence of the carbohydrate residue at position 5 does not permit the addition of a second atom of bromine to the carbon at position 5. Thus a 5-monobromo-derivative is formed. Desaturation of the 4,5-bond during the subsequent heating in ethanol may result in the elimination of either the bromine atom (Reaction 4) or the hydrogen atom (Reaction 5) from the carbon at the 5 position which would result in the formation of either uracil (Compound VII) or 5-bromouracil (Compound VIII). Since the linkage between the pyrimidine and the carbohydrate component has been found to be very labile after the saturation of the 4,5-double bond of the ring[29] the rupture of the linkage between the pyrimidine and the carbohydrate residue of the dialdehyde following bromination may proceed through reactions 3 or 3b, after the saturation of the 4,5-double bond. No detectable amount of the pyrimidine component, however, was obtained when the unknown nucleoside itself was treated with bromine. Thus, the presence of the dialdehyde or the dicarboxylic acid contrib-

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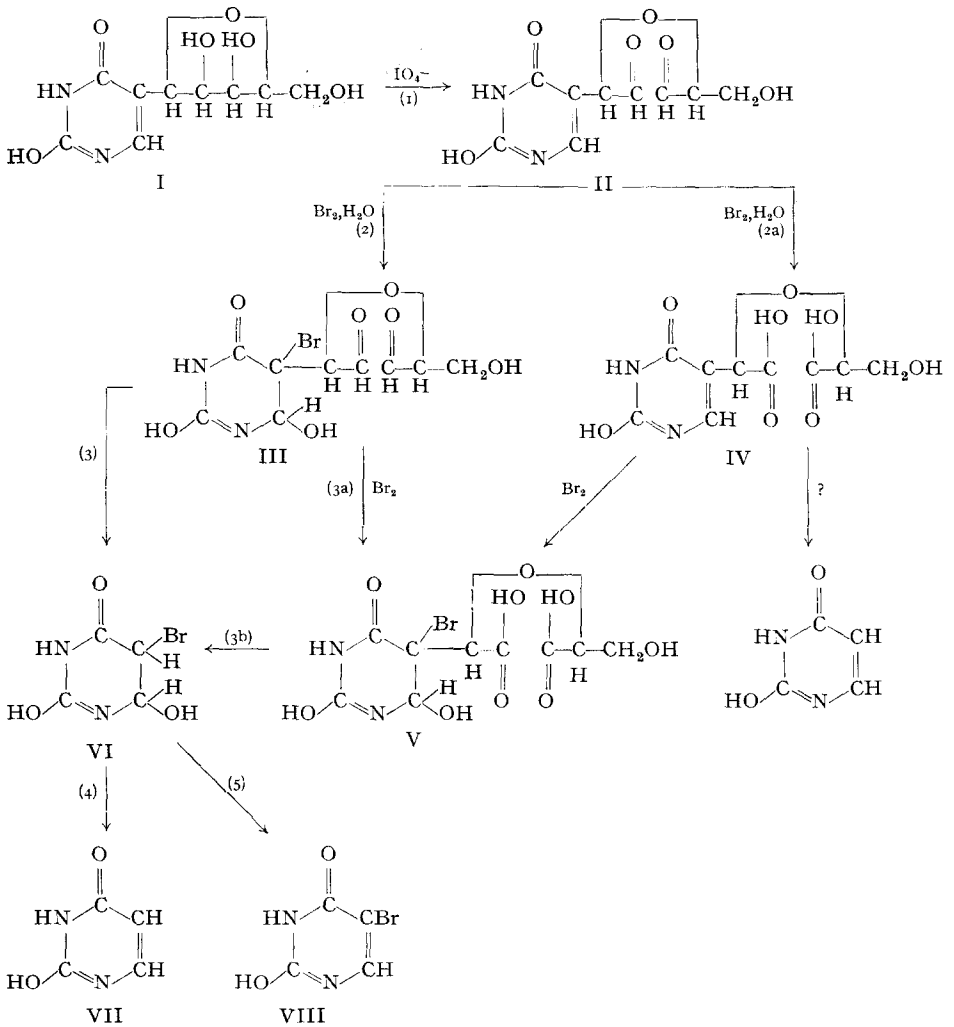
utes to the rupture of the linkage between the pyrimidine and carbohydrate residue (Reaction 3 or 3b). It is possible that the rupture of the bond between the oxidized residue of the carbohydrate and the pyrimidine component is essential for retaining the bromine atom.

SCHEME I



The postulate that the unknown nucleoside is uracil-5-riboside can also be used to interpret the results of the bromination experiments on the unknown nucleoside. In the bromination of uridine, 4-hydroxy-5,5-dibromohydrouridine was considered as the intermediate[22]. In the case of the unknown nucleoside, the presence of the carbohydrate at the 5 position of the ring would result in the formation of a 5-monobromoderivative as the only intermediate. In the subsequent boiling in ethanol, the bromine atom was preferentially eliminated. Thus a brominated derivative of the

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unknown nucleoside with a 4,5-double bond in the pyrimidine component could not be obtained. BAUDISCH AND DAVIDSON[30] described a series of reactions which involved the bromination of thymine in which the bromine atom at the 5 position of the pyrimidine ring could be readily eliminated.

It is to be noted at this time, however, that the selection of the 5 rather than the 4 position of the pyrimidine ring to be the linkage point between the pyrimidine and the carbohydrate components of the unknown nucleoside is based entirely on the comparison between the spectral data of 5-hydroxymethyluracil and those of the unknown nucleoside. The spectral data of 4-methyluracil and 5-methyluracil (thymine), as shown in Table V, are almost equally different from those of the unknown nucleoside. Furthermore experimental results discussed previously can well be interpreted on the assumptions that the carbohydrate residue is attached to the 4 position of the ring and that it interferes with the addition of a second bromine atom to the 5 position. Further work pertaining to the confirmation of the structure of this unknown nucleoside are reported in the next paper.

While the preparation of this manuscript was near completion, it was noted that COHN[31] reported recently that the unknown nucleotide had identical C, N and P content as uridylic acid and its alkaline spectrum was nearly identical with that of 5-hydroxymethyluracil. Although no detailed data were given by this author, these statements are in agreement with our findings on the properties of the nucleoside component of the unknown nucleotide. In contrast to the properties of the nucleoside, however, the unknown nucleotide was found to resemble uridylic acid in chemical stability and chromatographic behavior by this author. As discussed previously, the chemical stability and the chromatographic behavior of the unknown nucleoside are quite different from those of uridine. The unknown nucleoside, in fact, can be isolated from the commercial preparations of uridine by chromatographic methods.

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METHYLATION STUDIES ON VARIOUS URACIL DERIVATIVES AND ON AN ISOMER OF URIDINE ISOLATED FROM RIBONUCLEIC ACIDS

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SUMMARY

1. Various uracil derivatives including uridine and the recently isolated isomer of uridine were methylated with dimethylsulfate and the course of the reaction followed by isolating chromatographically and characterizing spectrophotometrically the products which were formed after various time intervals.

2. The methylation products of the isomer of uridine indicate that the 1, 2, 3 and 6 positions are not involved in glycosidic linkage and that the C-4 is much less likely than the C-5 to be the point of attachment of the sugar moiety, thus indicating that the isomer of uridine is 5-ribosyluracil.

INTRODUCTION

YU AND ALLEN[1] have shown that the new nucleoside of DAVIS AND ALLEN[2] contains uracil and is an isomer of uridine. Evidence that the carbohydrate moiety is ribofuranose has also been presented[3]. Consequently the major difference between uridine and its isomer must be the position of the linkage between the uracil and the ribose. In this regard it should be noted that the spectrum of the isomer shows the alkaline shift to longer wave lengths which is characteristic of uracil derivatives that are unsubstituted in position 3[3]. The O-2 and O-6 positions were thought unlikely to be

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