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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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involved in glycosidic bonds on the ground that the linkage was still intact after treatment with 1 *N* sulfuric acid at 100° for 1 h[1]; a condition under which oxygen glycosides are readily hydrolyzed[4]. As will be reported in a later paper, a riboside of 3-methyluracil was prepared in this laboratory and this substance was significantly different from the methylated isomer of uridine with regard to ultraviolet absorption spectra and chromatographic mobility thus suggesting that the N-1 position is not involved in the linkage to the sugar. However, while preparing the methyl derivative of the isomer of uridine chromatographic indications of the formation of more than one methylation product caused us to undertake a more detailed investigation of the course of the methylation of the isomer and of various other uracil derivatives. These experiments and the relationship of the results to the structure of the nucleoside constituted the substance of this report.

EXPERIMENTAL

Materials

The isomer of uridine was prepared from commercial uridine in accordance with the method of YU AND ALLEN[1]. Uridine was purchased from Schwarz Laboratories, dimethylsulfate from Eastman Kodak Company, 4-methyluracil from Nutritional Biochemicals Corporation, thymine and uracil from the California Foundation for Biochemical Research. 3-Methyluracil was graciously supplied by Dr. JOHN EILER who synthesized it by the procedure of HILBERT AND JOHNSON[5]. 1-Methyluracil was prepared by methylating uridine and treating the product with 12 *N* perchloric acid[6]. Spectral work was done by the use of a Beckman Model DU spectrophotometer.

Methods: N-Methylation of uracil, uridine, the isomer of uridine, 3-methyluracil, 1-methyluracil, 4-methyluracil and thymine

In all cases 50 μmoles of the uracil derivative was dissolved in 2.0 ml of distilled water. 200 μmoles (20 μl) of dimethylsulfate was added at the start of the experiment and again after 1 h. The reaction was carried out in a Beckman Model G pH Meter and pH 9.0 was maintained by the addition of 3 *N* sodium hydroxide from a micrometer controlled syringe. 15 μl-aliquots which contained 0.33 μmoles of uracil derivatives were taken at 0, ½, 1, 2 and 4 h, mounted on Whatman No. 1 filter paper and subjected to 12–18 h ascending flow of 86/14 (v/v) *n*-butanol–water solvent. The chromatograms were then dried and photographed (Fig. 1A, D, E, F and G); areas of interest were eluted and diluted appropriately for spectrophotometry.

Large scale preparation of the methylated isomer of uridine

Preparation of sufficient quantity of the methylated isomer of uridine for elemental analysis was accomplished by methylation of three 50 μmole-samples of the nucleoside. After 4 h of methylation the solutions were applied as three 6-in. streaks on Whatman No. 1 filter paper and chromatography carried out as previously described. The zones which correspond to spot A-4 (Fig. 1) were eluted, concentrated, and combined. Purification from paper blank materials was achieved by recrystallization from 100 % ethanol. The yield which was 20 mg was submitted for elemental analysis. O-methyl group was determined by the method of PREGL[7]. The methylated

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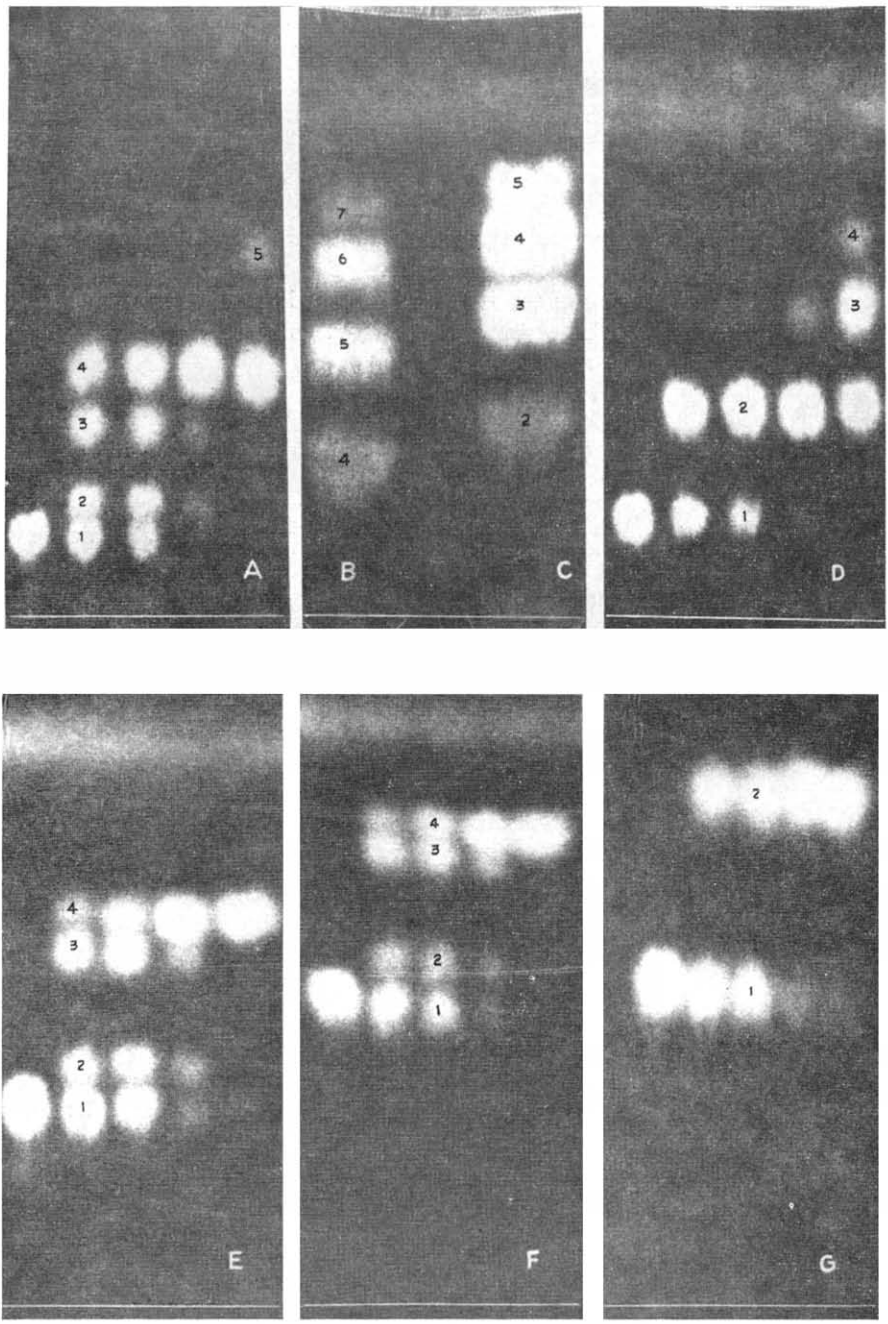

Fig. 1. Chromatography in 86/14 (v/v) Butanol–H_2O of methylated uracil derivatives. A. Isomer of uridine — light methylation; B. Isomer of uridine — heavy methylation; C. Uridine — heavy methylation; D. Uridine — light methylation; E. Uracil; F. 5-Methyluracil; G. 4-Methyluracil.

derivative of the isomer of uridine was sufficiently soluble in camphor to permit the determination of molecular weight by the RAST method to an accuracy of 90 %.

Extensive methylation of uridine and the isomer of uridine

After 4 h of methylation an additional 250 μl of dimethylsulfate was added to the uridine solution. The reaction was allowed to proceed for 3 more h after which 150 μl of the solution was applied to filter paper as a 1-in. streak. In order to conserve material the methylated isomer of uridine was treated in the following manner. About 10 μmoles of the substance which was obtained by evaporating the supernatant solution from the ethanol crystallizations was dissolved in 1.0 ml of water and treated with 100 μl of dimethylsulfate for 3 h. 100 μl of this solution was also applied to filter paper as a 1-in. streak. Chromatography (Figs. 1 B and C) and spectrophotometry were carried out as described in a foregoing paragraph.

RESULTS

Resolution of various products which were formed during methylation of the uracil derivatives was achieved by the chromatographic solvent. Uridine gave rise to four products (Figs. 1 C and D). Since the spectral ratios of areas C-2, 3, 4 and 5 are virtually identical (Table I), it is clear that N methylation occurred first and that only under considerably stronger conditions did appreciable methylation of the hydroxyl groups of the pentose occur. That areas C-3, 4 and 5 correspond to single, double and triple methylation of the carbohydrate moiety rather than the three isomeric singly methylated products is shown by the differences in R_F values and by the fact that in Fig. 1-D area 3 is much larger than area 4 while in Fig. 1-C the reverse relation obtains.

TABLE I

SPECTRAL AND CHROMATOGRAPHIC PROPERTIES OF METHYLATED URACIL DERIVATIVES

Uracil derivative	*Area No.*	R_F *butanol-H_2O*	*Spectral ratios*					
			pH 2			*pH 12*		
			250/260	*280/260*	*290/260*	*250/260*	*280/260*	*290/260*
Uridine isomer	A-2	0.15	0.54	1.03	0.38	0.62	0.69	0.08
Uridine isomer	A-3	0.26	0.77	0.38	0.06	0.57	3.28	3.22
Uridine isomer	A-4, B-4	0.33	0.56	0.89	0.27			
Uridine isomer	A-5, B-5	0.52	0.55	0.95	0.30			
Uridine isomer	B-6	0.65	0.56	0.95	0.32			
Uridine isomer	B-7	0.74	0.56	0.93	0.31			
Uridine	C-2, D-2	0.36	0.76	0.30	0.02			
Uridine	C-3, D-3	0.56	0.76	0.29	0.00			
Uridine	C-4, D-4	0.68	0.77	0.30	0.02			
Uridine	C-5	0.78	0.79	0.29	0.03			
Uracil	E-2	0.41	0.60	0.64	0.08	0.68	0.38	0.02
Uracil	E-3	0.61	0.86	0.14	0.02	0.39	3.63	3.12
Uracil	E-4	0.67	0.60	0.55	0.06			
5-Methyluracil	F-2	0.58	0.54	1.14	0.53	0.61	0.90	0.24
5-Methyluracil	F-3	0.77	0.69	0.50	0.09	0.65	3.01	3.50
5-Methyluracil	F-4	0.81	0.55	1.07	0.44			
4-Methyluracil	G-2	0.72	0.61	0.56	0.06	0.57	0.82	0.22

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Methylation of uracil gave rise to roughly equal quantities of two intermediates which were identified by their spectral ratios as 3-methyluracil (E-2) and 1-methyluracil (E-3)[4]. After 4 h of methylation only the final product, 1,3-dimethyluracil (E-4) was present. In order to investigate the possibility that demethylation or transmethylation between the 1 and 3 positions took place as well as to check whether O-methyl intermediates were formed, 1-methyluracil and 3-methyluracil were also subjected to methylation. In both cases only one product, 1,3-dimethyluracil, was detected.

Figs. 1 A and B show that methylation of the isomer of uridine resulted in six products. It is instructive to compare the ultraviolet absorption spectra of these substances to those of the methylation products of uracil (Fig. 2). The substances from areas A-2, A-3 and A-4 are related respectively to those of areas E-2, E-3 and E-4 in regard to absorption maxima and spectral ratios. The shifts in alkaline spectra relative to acid spectra are especially indicative. Substances from A-2 and E-2 both show shifts downward and slightly to the left while substances from A-3 and E-3 show pronounced shifts upward and to the right. The spectra of substances A-4 and E-4 are unaffected by the change in pH from 2 to 12 thus indicating that these substances do not contain dissociable hydrogen in this pH range. Therefore it can be concluded that substances A-2, A-3 and A-4 are nucleosides of 3-methyluracil, 1-methyluracil, and 1,3-dimethyluracil.

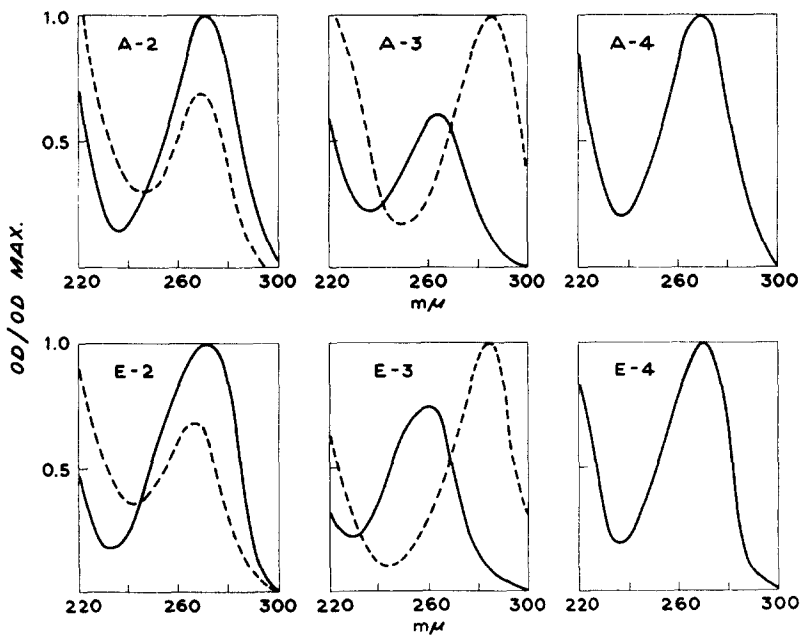

Fig. 2. Spectra of methylation products of the isomer of uridine, A–2, 3 and 4, and of uracil, E–2, 3 and 4. ——— pH 2, – – – – pH 12.

Elemental analysis of the substance eluted from A-4 gave results in satisfactory agreement with those calculated for 1,3-dimethyluracil nucleoside.

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	C	H	N	O–CH_3	MW
Experimental	47.80	5.90	10.30	1.5	290
Calculated	48.53	5.88	10.29	0	272

The small amount of O–CH_3 group found was thought not to be significant since the experimental procedure does not always measure O–CH_3 group to the complete exclusion of N–CH_3 group.

As was the case for uridine further methylation gave rise to products which for the reasons previously presented are thought to contain singly (B-5), doubly (B-6), and triply (B-7) methylated sugars.

Methylation of 5-methyluracil (Fig. 1-F) and 4-methyluracil (Fig. 1-G) resulted in strikingly different patterns. 5-Methyluracil gave rise to N-methyl products analogous to those formed from uracil and the isomer of uridine, but 4-methyluracil gave rise to only a single product. It is noteworthy that this product still possesses a dissociable hydrogen as is evidenced by the change in spectral ratios at pH 12 relative to pH 2. That this hydrogen is on the 3 position is indicated by the small but significant shift in ultraviolet absorption to longer wavelengths at pH 12. It is also worthy of note that the migration in butanol–water solvent of 4-methyluracil and its methylation product is related in a manner similar to that of uracil and 1-methyluracil.

DISCUSSION

The fact that methylation of the isomer of uridine gives rise to products analogous to those formed by the methylation of uracil proves quite conclusively that neither the 1 nor the 3 positions of uracil are involved in the linkage of the ribose to the uracil in the nucleoside. Also because of the lactim–lactam relationship of the oxygens of positions 2 and 6 to the nitrogens of positions 1 and 3, it follows that the 2 and 6 positions are not implicated in glycosidic bonds. The chance that the compound could be a dimer is discounted on the basis of the molecular weight of the methylated derivative. Consequently the ribose must be linked to either the 4 or the 5 position of uracil by means of a C–C bond. Of these two there are several reasons for preferring the 5 position. (1) If biosynthesis of the nucleotide follows the same pathway as biosynthesis of uridylic acid, the 4 position is blocked by the carboxy group of orotic acid. (2) The chemical reactivity of the 5 position is generally thought to be greater than that of the 4 position. (3) Another 5-substituted pyrimidine derivative, the vitamin thiamine, exists in nature. Methylation studies also point to the 5 position since 5-methyluracil can be completely methylated to 1,3-dimethylthymine via the 1- and 3-methyl intermediates while 4-methyluracil can only be singly methylated to 1,4-dimethyluracil. If, then, the much larger carbohydrate group were attached to the 4 position, methylation of the 3 position would be obstructed to an even greater extent. This, however, is not in accord with the facts since methylation of the new nucleoside shows that the 1 and 3 positions are about equally accessible to the attack of dimethylsulfate. Consequently the weight of evidence bears heavily on the 5 position as the point of attachment of the carbohydrate moiety and the work reported in this and the preceding paper[1] leads us to conclude that the isomer of uridine is 5-ribosyluracil.

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ACKNOWLEDGEMENT

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NUCLEOSIDE MONOPHOSPHATE KINASES

I. TRANSPHOSPHORYLATION BETWEEN ADENOSINE TRIPHOSPHATE AND NUCLEOSIDE MONOPHOSPHATES

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SUMMARY

1. The nucleoside monophosphate kinase catalyzing the ATP–UMP reaction has been purified 40–45-fold from an extract of calf liver acetone powder. The ATP–CMP kinase followed the fractionation closely but the ATP–AMP kinase was partially removed while the ATP–GMP kinase and the kinases for reactions between nucleoside triphosphate and AMP were completely removed.

2. Some of the properties of this ATP–nucleoside monophosphate kinase preparation are presented.

INTRODUCTION

The synthesis of nucleoside di- and triphosphates from nucleoside monophosphates is catalyzed by enzymes which transfer phosphate from one nucleotide to another.

The following terminology will be used throughout the paper: AMP, ADP, ATP = adenosine 5′-mono; di- and triphosphates; IMP, IDP, ITP = inosine 5′-mono, di- and triphosphates; GMP, GDP, GTP = guanosine 5′-mono, di- and triphosphates; UMP, UDP, UTP = uridine 5′-mono-, di- and triphosphates; CMP, CDP, CTP = cytidine 5′-mono, di- and triphosphates; EDTA = ethylene diamine tetraacetate; DPNH = reduced diphosphopyridine nucleotide.

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