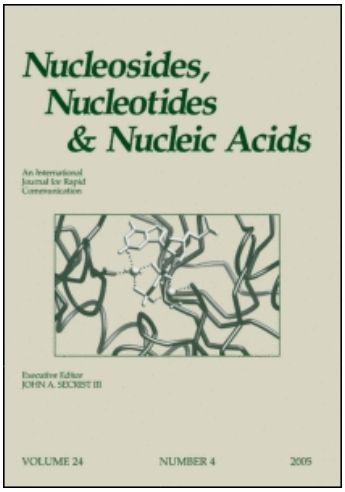

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Isolation and Characterization of 5-Carbamoylmethyluridine and 5-Carbamoylmethyl-2-Thiouridine from Human Urine

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ISOLATION AND CHARACTERIZATION OF 5-CARBAMOYLMETHYLURIDINE AND 5-CARBAMOYLMETHYL-2-THIOURIDINE FROM HUMAN URINE[a]

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ABSTRACT

Two new modified uracil nucleosides, 5-carbamoylmethyuridine (ncm^5U, I) and 5-carbamoylmethyl-2-thiouridine (ncm^5s^2U, II) were isolated from a 24 hr collection of a normal human urine. The structures were assigned on the basis of UV, NMR and mass spectral data and confirmed by comparison of the spectral data and HPLC mobilities with those of authentic samples. On the basis of experimental data it appears possible that 5-carbamoylmethyl-2-thio-uridine (ncm^5s^2U, II) may be a degradation product produced from a labile precursor by the chemical treatments during the isolation procedure. However, the other nucleoside (ncm^5U,I) certainly appears to be of metabolic origin and was also found in the urines of one chronic myelogenous leukemia and one lung carcinoma patient.

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a) This study was presented in part at the 36th Conference of the American Society for Mass Spectrometry and Allied Topics, June, 1988, San Franciso, CA.

b) Deceased.

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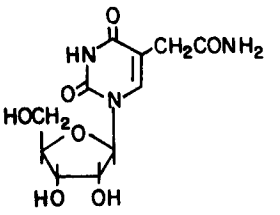
Abbreviations used are: tRNA-transfer ribonucleic acid, TMS-trimethylsilyl, RP-HPLC - reverse phase high performance liquid chromatography, EI - electron impact, cm^5U-5-carboxymethyluridine, mcm^5U-5-methoxycarbonylmethyluridine, cm^5s^2U-5-carboxymethyl-2-thiouridine, mcm^5s^2U-5-methoxycarbonylmethyl-2-thiouridine, t^6A-9-β-D-ribofuranosyl-[N(purin-6-yl)carbamoyl]-l-threonine, C-cytidine, acp^3u-3-(3-amino-3-carboxypropyl)uridine, AICR-aminoimidazole carboxamide riboside, α-4-PCNR & β-4-PCNR-9-α-D-(or β-D)-ribofuranosyl-pyridin-4-one-3-carboxamide, H x ^{7}R-7-β-D-ribofuranosyl hypoxanthine, m^3U-3-methyluridine, m^1I-1-methylinosine, m^1G-1-methylguanosine, dI-5′-deoxyinosine, dms^5OA-5′-deoxy-5′-methylthioadenosine sulfoxide, $m^2{}_2G$-N_2-dimethylguanosine, ψ-ψ-uridine, A-adenosine, I-Inosine, CML-chronic myelogenous leukemia, mam^5s^2U-5-methylaminomethyl-2-thiouridine, ncm^5U-5-carbamoylmethyluridine, ncm^5s^2U-5-carbamoylmethyl-2-thiouridine, UV-ultraviolet, NMR-nuclear magnetic resonance, HPLC-high performance liquid chromatography, GC-MS-gas chromatography-mass spectrometry.

INTRODUCTION

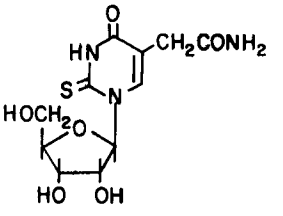
For a number of years we have been interested in disease related nucleic acid metabolites in human body fluids, particularly urine[1]. As a part of this program, we have been looking for the presence of modified nucleosides and bases derived from cellular anabolic and catabolic processes in the urines of cancer patients and normal human subjects[2]. Of more than 65 modified nucleosides present in mammalian tRNA, 21 have so far been isolated from human urine. Included among these are the anticodon adjacent modified nucleosides such as t^6A[3], 1-methylinosine[4] and others. Some of the modified nucleosides, such as N^2-dimethylguanosine[4], derived from tRNA catabolism, are excreted in elevated amounts in the urines of cancer patients[5]. Other modified nucleosides that appear in human urine such as N^6-succinyladenosine[6], 7-β-D-ribofuranosylhypoxanthine[7], 5′-deoxyinosine[8], 1-β-D-[9] and 1-α-D-ribofuranosylpyridin-4-one-3-carboxamide[10] originate in sources other than nucleic acids and some of these have also been found at elevated levels in the urines of patients with neoplastic diseases. These and other studies[11,12] indicate that such modified nucleosides may be useful as markers in cancer diagnosis and also for the assessment of tumor burden and therapeutic effectiveness in such patients.

Extension of these studies to immunodeficiency disorders such as AIDS[13] has proven to be of similar value. This paper deals with the isolation and characterization of two novel modified nucleosides, 5-carbamoylmethyluridine (ncm^5U, I) and 2-thio-5-carbamoylmethyluridine (ncm^5s^2U, II) from normal human urine. Nucleoside I (ncm^5U) was also isolated from the urine of one patient with chronic myelogenous leukemia and one lung carcinoma patient.



I

5-CARBAMOYLMETHYLURIDINE



II

5-CARBAMOYLMETHYL-2-THIOURIDINE

MATERIALS AND METHODS

Neutral charcoal (Norit) was purchased from Fisher Scientific Co., and Celite 545 was obtained from Johns-Mansville Co. DEAE cellulose (DE-23) and AG1-X8 formate (200-400 mesh) anion exchange resin were obtained from Whatman and Bio-Rad Labs., respectively. Deuterium oxide (99.96 atom % D) was purchased from Aldrich Chemical Co. Glass distilled methanol was obtained from Burdick & Jackson. 5-Methyl-2-thiouridine, 5-hydroxymethyuridine and 5-methyoxyuridine were purchased from Sigma Chemical Co., 5-methyuridine was obtained from Mann Research and Affi-gel 601, affinity gel, was purchased from Bio-Rad Laboratories. The Silylating agent bis(trimethylsilyl)trifluroacetamide (BSTFA) containing 0.1% trimethylchlorosilane (TMCS) and pyridine were purchased from Regis Chemical Company. Authentic ncm^5U, cm^5U and mcm^5U were a generous gift from Dr. John F. Fissekis from Sloan-Kettering Institute of Cancer Research, New York, NY and mcm^5s^2U and mam^5s^2U were kindly provided by Dr. A. Malkiewicz, Institute of Organic Chemistry, Zwirki, Poland.

Ultraviolet spectrophotometry: Ultraviolet (UV) spectra were recorded on a Cary 219 spectrophotometer which was zeroed with water using the auto baseline feature.

Nuclear magnetic resonance (NMR) spectrometry: NMR spectra were determined on a Bruker WP-200 (200 MHz) spectrometer by utilizing the Fourier-transform/quadrature phase detection mode. Sample temperatures were maintained at 30° with the BVT-2000 temperature controller of the WP-200 spectrometer. The chemical shifts reported here are given in (δ) ppm, and are measured from internal TSP (sodium 3-trimethylsilylproprionate-2,2,2,2-d4).

Mass spectrometry (MS) and gas chromatography-mass spectrometry (GC-MS): Low resolution mass spectral studies and GC-MS studies were carried out using a Finnigan 4000 quadrupole instrument interfaced to an INCOS data system.

All samples were analyzed as their trimethylsilyl (TMS) derivatives which were formed by heating approximately 0.02 A_{263} units (~ 800 ng) of vacuum dried material with anhydrous pyridine and bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing a 0.1% trimethylchlorosilane (TMCS) (1:1, 4 μL) in a sealed melting point capillary tube at 78.5°C for 1 hour.

The fused silica capillary column (30 meters long, 3% SE-30, 25μ film thickness) was interfaced directly into the ion source allowing for maximum sensitivity. Samples were injected in the splitless mode (injector temp. 280°C) and the column oven was temperature programmed from 80°C (2 min. hold) to 280°C (10 min. hold) at a rate of 10°C/min. for each run. All mass spectral information was obtained at an ionizing voltage of 70 eV.

Chromatography

Reversed phase high performance liquid chromatography (RP-HPLC): RP-HPLC was carried out as discussed previously[2]. The following RP-HPLC solvent systems were used for coinjection studies: A) isocratic, 5% methanol in water; B) isocratic, 5% methanol in 0.1 M ammonium acetate buffer, pH 3.8; C) gradient, 0 → 30% methanol in water in 20 min.; D) isocratic, 0.1 M ammonium acetate buffer, pH 4.7; E) gradient 0 → 10% methanol in 0.1 M ammonium formate buffer, pH 4.3, in 20 min.; F) gradient 0 → 30% methanol in water in 30 min.; G) gradient 0 → 20% methanol in water in 20 min.; H) isocratic, 10% methanol in water; I) gradient 0 → 25% methanol in 0.1 M ammonium acetate

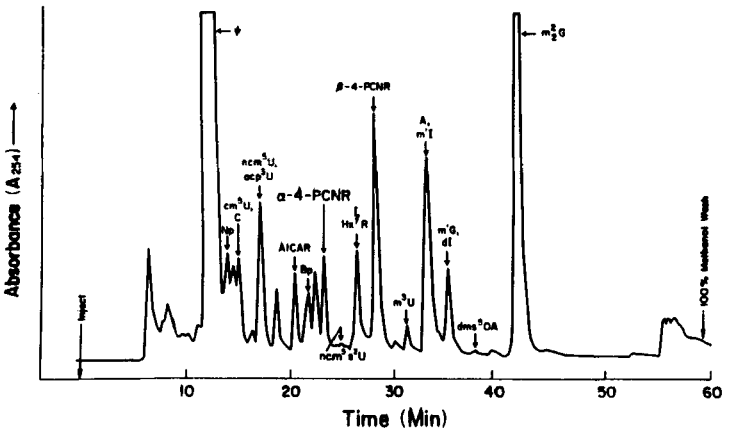


Fig. 1. Preparative RP-HPLC of urinary ribonucleosidic fraction on Zorbax column.

buffer, pH 7.0, in 30 min.; J) gradient 0 → 20% methanol in 0.1 M ammonium formate buffer, pH 4.3, in 30 min. All samples were run at a flow rate of 3 ml/min. at 25°C.

Isolation of Unknown Urinary Nucleoside I and Nucleoside II

Both nucleosides were isolated from the 24 hr urine collection of a normal male human volunteer by the procedure described previously[2, 14]. The urinary ribonucleosidic fraction isolated from DEAE-cellulose-borate column with 0.7 M boric acid was chromatographed on a Zorbax preparative RP-HPLC column using a gradient elution of 0 → 25% methanol in 0.1 ammonium acetate buffer, pH 7.0, for 1 hr at a flow rate of 8ml/min. Unknown urinary nucleoside I was isolated from the peak eluting at 18 min. and purified on a semi-preparative Ultrasphere ODS C_{18} RP-HPLC column eluting at 20 min. in water (240 μg; Fig. 1). Nucleoside II was isolated from the peak eluting at 25 min. and purified on an Ultrasphere ODS C_{18} RP-HPLC column eluting at 12 min. with 10% methanol in water (25 μg; Fig. 1). Nucleoside I was isolated similarly from a 24 hr urine collection of a CML (160 μg) and lung carcinoma patient (320 μg).

Preparation of (ncm^5s^2U)

To 8.0 A_{276} units of authentic mcm^5s^2U in 200 μl of water 5 ml of aqueous NH_4OH was added and the solution reacted at 25°C for 18 hrs[15]. The mixture was evaporated 3 times from methanol,

redissolved in water (1.0 ml) and purified on a semi-preparative Ultrasphere RP-HPLC ODS C_{18} column eluting with 10% methanol in water. The new amide product, ncm^5s^2U, was well resolved (11.4 min.) from the starting material (38.11 min.) and isolated in 75% yield.

Conversion of (mcm^5s^2U) to (cm^5s^2U)

The standard mcm^5s^2U (1.0 A_{276} units) was reacted with 0.5 ml of 0.5 M NaOH for 24 hrs at 25°C[15, 16]. The mixture was neutralized with 30 µl of acetic acid to pH 4.8 and purified on a semi-preparative Ultrasphere ODS C_{18} RP-HPLC column with a gradient elution of 0 → 30% methanol in water in 30 min. at a flow rate of 3 ml/min. The original ester eluted at 29.54 min. The new product, cm^5s^2U, eluting at 8.79 min., was isolated in good yield (80%).

Stability of mcm^5U and mcm^5s^2U in the preparative isolation procedure

The standard mcm^5U (1.0 A_{267} unit) was incubated in 1.5 ml of 2N NH_4OH in 50% aqueous ethanol for 48 hrs at room temperature. The reaction mixture was evaporated to dryness, redissolved in 0.1 M ammonium acetate buffer (pH 7.0) and injected onto a semi-preparative Ultrasphere ODS C_{18} RP-HPLC column. The products were fractionated by a gradient elution of 0 → 25% methanol in 0.1 M ammonium acetate buffer (pH 7.0) in 25 min. at a flow rate of 3 ml/min. and identified by comparing their elution positions and coinjection behavior with the appropriate standards. The synthetic mcm^5s^2U was reacted similarly but incubated for 24 hrs and fractionated on the same RP-HPLC column with 10% methanol in water as mobile phase.

Detection of unknown urinary nucleoside (I) in a fraction isolated from a boronate affinity gel column

The coplanar cis-diol compounds (functional group of the ribonucleosides) were separated from other urinary substances by a one-step boronate gel affinity micro chromatography procedure developed by Gehrke and co-workers[17]. The use of a larger column bed (1.0 x 9.0 cm) enabled 25 ml of urine to be processed. The column packing, urine preparation and chromatographic procedures were virtually identical to those reported by Gehrke and co-workers[17], except that a corresponding increase in buffer volume was necessary for the scale-up procedure. The ribosides were eluted from the column by 150 ml of 0.1 N formic acid. The acid fraction was concentrated, neutralized, then resolved into more than 30 peaks when reinjected onto a semi-preparative Ultrasphere ODS C_{18} RP-HPLC column. The eluting

buffer was a gradient of 0 → 30% methanol in 0.01 M ammonium acetate buffer (pH 4.4) in 60 min. at a flow rate of 3 ml/min. Peaks eluting at the retention times corresponding to the predetermined elution position of the selected standards (retention time ± 5 min.) were collected individually and the UV absorption spectra at pH 4.4 were obtained. These selected peaks were injected individually or coinjected along with appropriate standards and rechromatographed on the semi-preparative ODS C_{18} RP-HPLC column in several buffer systems. The collective mobility characteristics confirmed or excluded homogeneity between the natural urinary material and the synthetic standard. The predetermined eluting positions of standards, including those corresponding to the two urinary unknowns (ncm^5U and ncm^5s^2U), the corresponding esters mcm^5U and mcm^5s^2U and a possible acidic degradation product cm^5U, were obtained by adding authentic standards to an aliquot of the riboside formic acid fraction and chromatographing this spiked aliquot in an identical RP-HPLC system. Comparison of the retention times and increased peak areas with an "unaltered" urinary chromatogram identified the peaks of interest.

RESULTS

The ultraviolet (UV) spectra of unknown urinary nucleoside I (Fig. 2a) is characteristic of a number of 5-alkyl substituted uridines found in tRNA[18] and is identical to the UV absorption spectra of authentic 5-carbamoylmethyluridine (ncm^5U) (Fig. 2b).

The NMR spectrum of nucleoside I in D_2O is consistent with a 5-substituted uridine. The lone aromatic resonance signal at 7.84 ppm is attributed to the C_6-H proton of the uracil base suggesting the substitution of the C_5-H proton. The doublet centered at 5.96 ppm (J = 4.3) could be assigned to an anomeric proton while the multiple resonance signals at 3.5 - 4.5 ppm are characteristic of unsubstituted ribose protons. The single upfield resonance at 3.35 ppm can be assigned to the methylene protons of the 5-substituent. In all, NMR spectra of I is identical to those of the authentic ncm^5U: δ 7.84 (s, 1, 6-H), 5.96 (d, 1, J1′, 2′ = 4.3 Hz, 1′-H), 3.5 - 4.5 (m, 3, 3′,4′,5′-H); 3.35 (s, 2 CH_2) ppm (Table I).

Structural assignment was further supported through mass spectrometry studies on the unknown urinary nucleoside I. A penta trimethylsilyl derivative of I gave a $(M-CH_3)^+$ ion peak at m/z 646 (Fig. 3a).

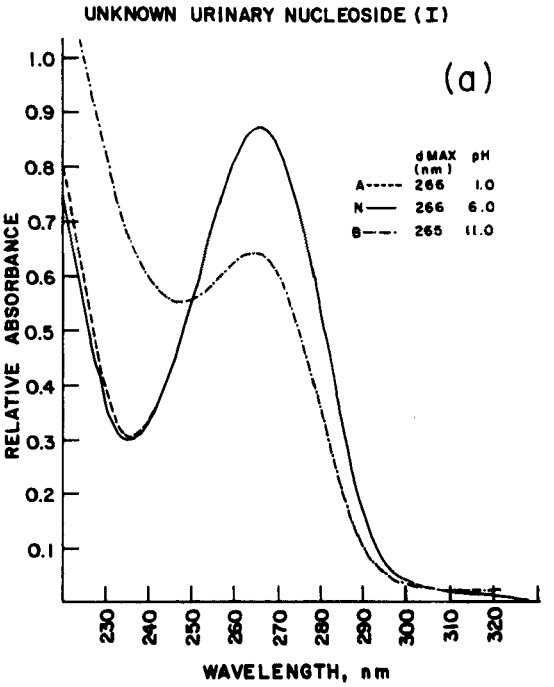


Fig. 2. UV spectra of unknown Nucleoside I and authentic ncm^{5}U.

This data is in agreement with the mol. ion peak of the penta TMS derivative of the nucleoside I at m/z 661. The fragment ion at m/z 546 (M-115) represents the loss of a portion of the side chain (CON(TMS)) while the fragment ion at m/z 386 arises from the cleavage of the glycosidic bond[19, 10]. The ions at m/z 349, 243 and 169 confirm the presence of an unmodified ribose sugar in the molecule[20]. More importantly, the GC/MS spectrum of the penta trimethylsilyl derivative of authentic ncm^{5}U (Fig. 3b) is identical to that of the unknown urinary nucleoside I, thus supporting ncm^{5}U as the unknown nucleoside I. Their GC retention times were also identical (retention time for nucleoside I – 25.52 min.). In high resolution FAB mass spectrometry the $(M+H)^+$ ion for nucleoside I was at 302.0969 daltons which compares well with calculated value for $C_{11}H_{16}N_3O_7$ at 302.0988 daltons.

Comparison of retention times of the naturally occurring nucleoside I and authentic ncm^{5}U by RP-HPLC further supported the identity of the two materials. The elution of the coinjected mixture as a

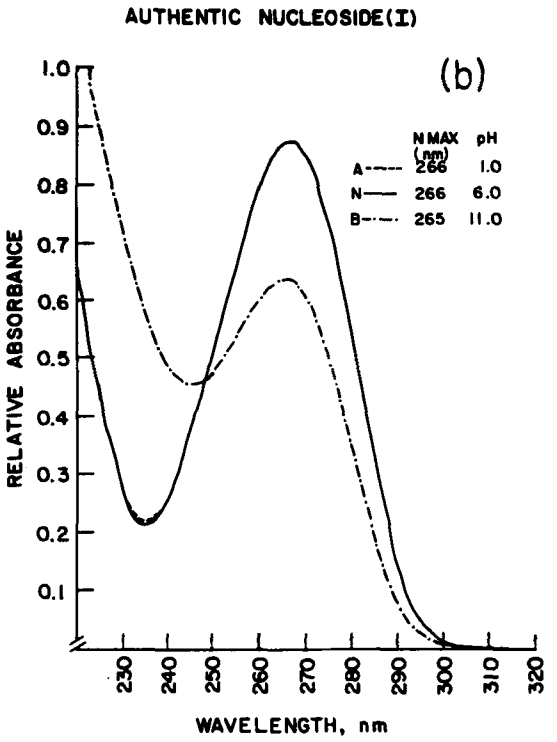


Fig. 2 Continued

Table I

Chemical Shifts (δ) and J Values (Hz) for Unknown Urinary Nucleoside I and Authentic 5-Carbamoylmethyluridine in D_2O

Compounds	C6-H	C1'-H	C3'-H,C4'-H,C5'-H	CH_2
Unknown urinary nucleoside I	7.84 (s)	5.96(4.3)	3.5-4.5(m)	3.35(s)
Authentic 5-carbamoylmethyl-uridine	7.84(s)	5.96(4.3)	3.5-4.5(m)	3.35(s)

single homogenous peak in five solvent systems confirmed the identity of the urinary nucleoside I with ncm^{5}U (Table 2). Similarly coinjections with 5 other authentic 5-alkyl substituted uridines found in tRNA (as listed in Materials) excluded these as the unknown urinary nucleoside I. Cumulatively, the physico-chemical data unequivocally identify I as the 5-carbamoylmethyluridine (ncm^{5}U).

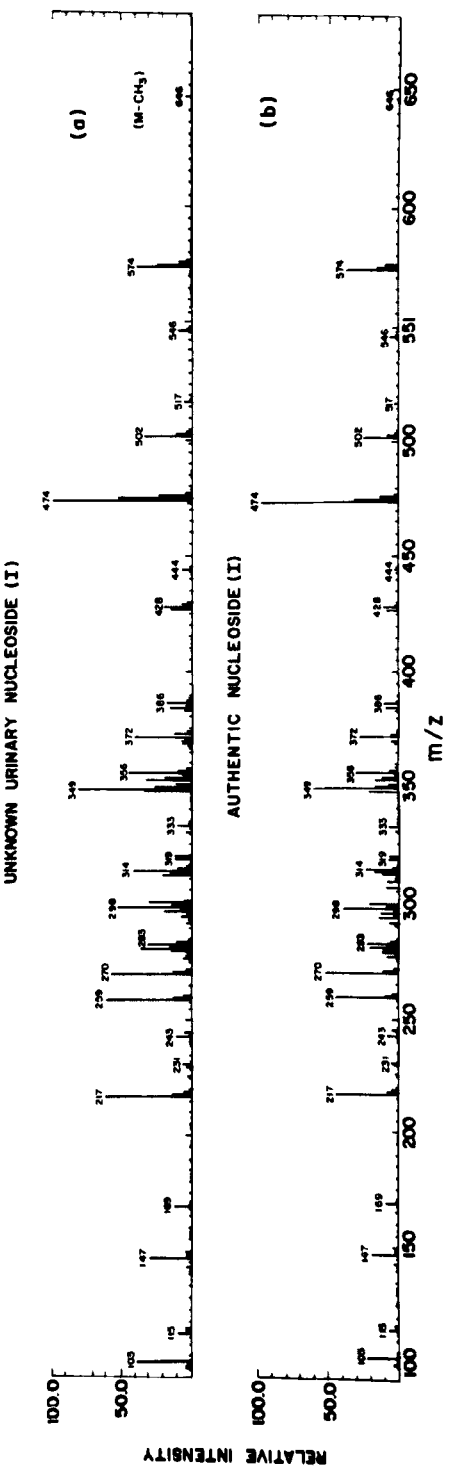


Fig. 3. Mass spectra of Penta TMS derivatives of unknown urinary nucleoside I (a) and authentic ncm^{5}U (b).

Table 2

Comparison of RP-HPLC Retention Time (Min.) of Unknown Urinary Nucleoside (I) and Authentic 5-Carbamoylmethyluridine (ncm^5U)

	Solvent System*				
Compound	A	B	C	D	E
Unknown urinary nucleoside (I)	9.43	7.95	14.63	21.41	16.60
Authentic 5-carbamoyl-methyluridine (ncm^5U)	9.47	7.95	14.71	21.25	16.57
Coinjected mixture of (I) and authentic standard	9.40	7.99	14.81	21.27	16.40

*see Materials and Methods section for solvent.

As for nucleoside II, its UV absorption spectrum is unique and similar to the spectra of a number of 5-alkyl 2-thiouridines isolated from tRNA[21-23] (Fig. 4a) and is identical to the UV absorption spectrum of the authentic ncm^5s^2U (Fig. 4b).

In GC-MS studies, the only peak relating to nucleoside II was a peak derived from the tetra TMS derivative of a dehydration product from nucleoside II. The mass spectrum had the highest mass peak at m/z 572 (Fig. 5a). This appears to be the $(M-CH_3)^+$ ion of the dehydration product of II, the tetra TMS derivative of which would show a mol. ion peak at m/z 587. Authentic ncm^5s^2U silylated under identical conditions also showed the same peak with the highest mass ion at m/z 572 (Fig. 5b). A peak corresponding to the tetra TMS derivative of the dehydration product from ncm^5U(I) was also observed in the GC chromatogram of penta TMS derivative of ncm^5U and the same phenomenon has been observed by us previously in other aromatics containing a carboxamide substituent side chain[9]. The dehydration appears to occur during silylation by extracting a molecule of water from the $-CONH_2$ moiety. The peaks at m/z 348, 245 and 169 indicate an unmodified ribose sugar[20]. The EI GC-MS spectra as well as the GC retention times for both nucleosides are identical supporting the molecular structure for the underivatized nucleoside II.

Furthermore, a comparison of RP-HPLC mobilities verify the identity of the naturally occurring nucleoside II with the authentic ncm^5s^2U. Retention times and elution profiles of both individual and

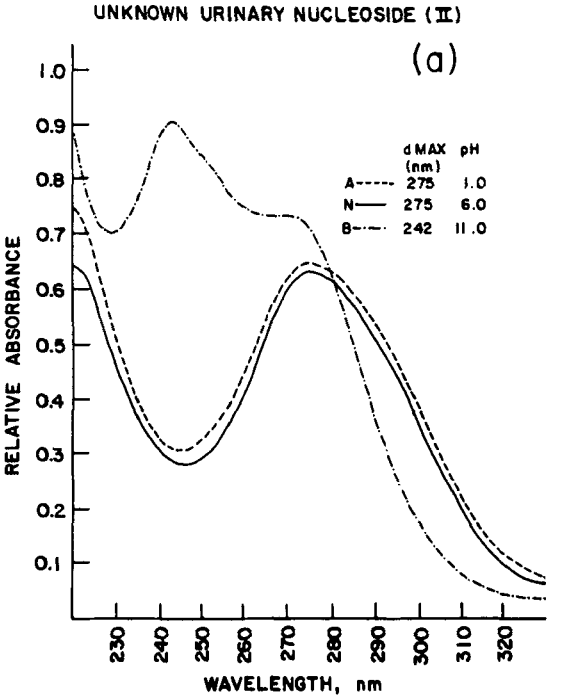


Fig. 4. UV spectra of unknown urinary nucleoside II (a) and authentic ncm^{5}s^{2}U (b).

coinjected mixtures were identical in five solvent systems (Table 3). In addition, RP-HPLC retention times and coinjections excluded mam^{5}s^{2}U, mcm^{5}s^{2}U, cm^{5}s^{2}U and 5-methy-2-thiouridine as the structures for the unknown urinary nucleoside II. Nucleoside II is thus identified as 5-carbamoylmethyl-2-thiouridine (ncm^{5}s^{2}U).

It is known that the conversion of the methyl ester, mcm^{5}U to the corresponding amide ncm^{5}U is possible in an alkaline environment[18]. In the initial desalting step of the preparative isolation procedure, 2N NH_4OH in 50% aqueous ethanol was used to remove the urinary nucleosides from the charcoal-celite column. In order to determine if these two unknown amide nucleosides isolated from the urine were genuine metabolites or transformed products, the parent methyl ester analogues (mcm^{5}U and mcm^{5}s^{2}U) were incubated in the alkaline media (pH 11.5). After two days the mcm^{5}U reaction mixture was chromatographed on a RP-HPLC column. The original ester, eluting at 24.1 min., was no longer

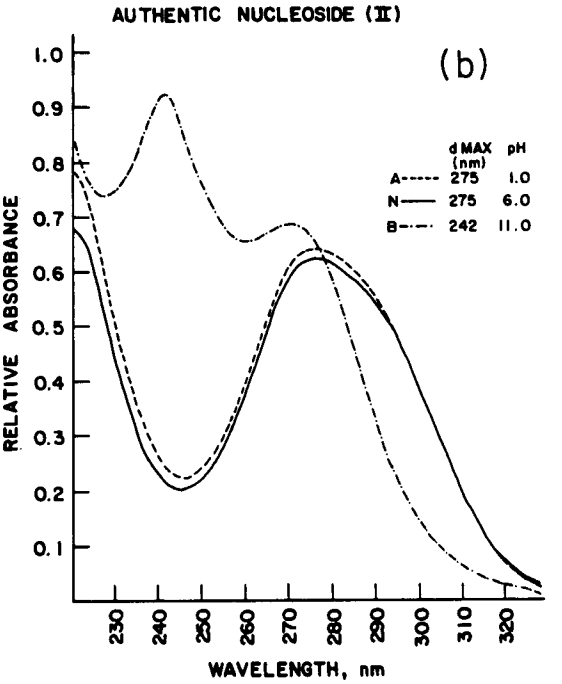


Fig. 4 Continued

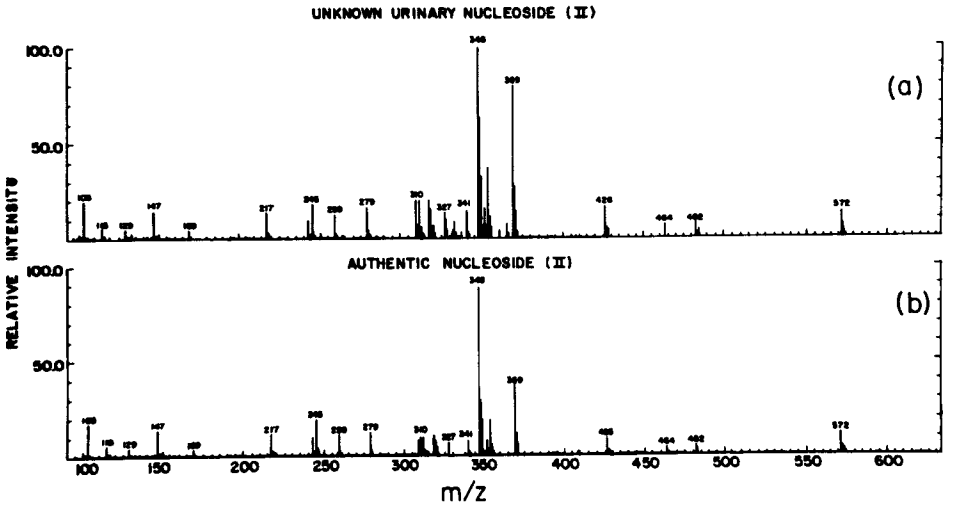


Fig. 5. Mass spectra of silylated unknown Nucleoside II (a) and authentic ncm^5s^2U(b).

Table 3

Comparison of RP-HPLC Retention Time (Min.) of Unknown Urinary Nucleoside (II) and Authentic 5-Carbamoylmethyl-2-thiouridine (ncm^5s^2U)

	Solvent System*				
Compound	F	G	H	I	J
Unknown urinary nucleoside (II)	18.83	19.73	11.91	22.11	25.45
Authentic 5-carbamoylmethyl-2-thiouridine	18.76	19.79	12.00	22.08	25.26
Coinjected mixture of (II) and authentic standard	18.75	19.76	11.89	22.00	25.20

*see Materials and Methods section for solvent.

detected. Two new reaction products, more polar than the starting material were detected, eluting at 11.8 min. and 15.2 min. These were identified as the acid (cm^5U) and the amide (ncm^5U) analogues respectively. As expected nucleoside I coeluted with the amide ncm^5U (15.2 min.).

The ester (mcm^5s^2U) was incubated under similar conditions and when applied to the RP-HPLC column could be detected (greatly reduced) eluting at 37.3 min. in 10% methanol in water. As with the uridine methyl ester, two new polar reaction products were identified as cm^5s^2U (5.2 min.) and ncm^5s^2U (10.8 min.) by coinjection with the standards. Nucleoside II coeluted with the amide ncm^5s^2U (10.8 min.).

Having demonstrated the labile nature of the precursor methyl ester metabolites in our isolation procedure, an attempt to isolate the urinary nucleosides by a milder isolation technique was initiated. In the micro procedure developed by Gehrke[17], co-planar cis-diol compounds such as ribonucleosides are immobilized as boronate complexes on an affinity gel column while other compounds are washed away (pH 8.8). The purified ribonucleosides are subsequently eluted from the column with dil. formic acid and using these conditions about 100 A_{260} units of cis-diol material from a 25 ml urine sample were collected.

The methyl ester precursor standards of both unknowns (mcm^5U and mcm^5s^2U) were isolated without any decomposition when subjected to this isolation procedure. Isolation, therefore, of the

unknowns I and II by this procedure would offer persuasive evidence supporting their presence in the urine as genuine metabolites rather than decomposition products. First the peak positions of the nucleoside I and II in the RP-HPLC chromatograms of the urinary formic acid fraction were determined by comparing the chromatograms of the "spiked" and unaltered ribosidic aliquots (see Methods section). For an aliquot "spiked" with authentic ncm^{5}U the peak for nucleoside I eluting at 15.8 min. increased significantly in area in the "spiked" urinary chromatogram. This agreed with the expected retention time for ncm^{5}U and conclusively identified the urinary peak of interest. The remaining pool of the formic acid urinary riboside fraction was chromatographed identically and the peak eluting at 15.8 min. was collected and its UV absorption spectrum obtained (λ_{max} 267 nm) in 0.01 M ammonium acetate buffer (pH 4.4). This sample was concentrated and coinjected with the authentic standard ncm^{5}U on RP-HPLC in four discriminating solvent systems which identified the urinary metabolite eluting at 15.8 min. as ncm^{5}U (nucleoside I). No methyl ester precursor (mcm^{5}U) could be detected under these conditions. A similar approach to detect nucleoside II (ncm^{5}s^{2}U) or its methyl ester (mcm^{5}s^{2}U) or acid (cm^{5}s^{2}U) proved unsuccessful. This established that nucleoside I (ncm^{5}U), isolated from the formic acid urinary fraction is a genuine metabolite present in urine rather than a decomposition product.

DISCUSSION

Nucleoside I has been isolated from the 24 hr urine collection of a normal male volunteer (240 μg), one CML (160 μg) and one lung carcinoma (320 μg) patient. As discussed above, it has been identified as ncm^{5}U by UV, NMR and GC-MS spectral data and by comparisons of its HPLC mobilities with those of the authentic standard. This nucleoside was first isolated from the alkaline hydrolysate of tRNA from Bakers yeast and wheat embryo[18] and in the RNase T1 digest of tRNA^{Pro} from T. utilis[24] and tRNA^{Val} from yeast cells[19] where it was shown to occupy the first position (Wobble) of the anticodon region. The earlier work suggests the possible origin of ncm^{5}U as a degradation product of the labile methyl ester (mcm^{5}U) generated during the alkaline hydrolysis isolation. Our preparative isolation technique also requires the use of an alkaline medium in the chromatographic fractionation of the nucleosides. A blank experiment on the stability of the synthetic mcm^{5}U in this alkaline medium demonstrated the labile nature of the methyl ester under these conditions. Both the acid (cm^{5}U) and amide analogues (ncm^{5}U) were generated from mcm^{5}U under these conditions.

In an effort to clarify the origin of urinary nucleoside I, a milder isolation procedure utilizing boronate affinity gel chromatography was attempted. The nucleosides were removed from a 25 ml aliquot of urine in less than 8 hours and were exposed to the maximum pH of 8.8 for less than 2 hrs. The synthetic standard methyl ester, mcm^{5}U, was shown to be stable under these conditions.

Nucleoside I was detected successfully in a RP-HPLC chromatogram from the urinary aliquot obtained by the milder isolation procedure by mobility and co-elution comparisons with synthetic standard ncm^{5}U. The ester mcm^{5}U, in contrast, was not found in this aliquot. This implies the absence or undetectable levels of this compound in the urine. Therefore, mcm^{5}U as a major source of I appears unlikely, while the detection of I in the same sample provides convincing evidence for its natural occurrence in urine.

Nucleoside II, identified as ncm^{5}s^{2}U, was isolated from one normal urine in extremely low amounts (25 μg). The isolation of this nucleoside from another biological source has not been reported previously. The corresponding methyl ester mcm^{5}s^{2}U, however, has been isolated from yeast tRNA[22] and tRNA Glu[25] from Bakers yeast. As with I, the origin of nucleoside II as a decomposition product from the labile methyl ester is a possibility. Here too, synthetic mcm^{5}s^{2}U is labile in the alkaline media. Unlike I, however, we were unable to detect the urinary nucleoside II (ncm^{5}s^{2}U) or the parent methyl ester (mcm^{5}s^{2}U) in the riboside fraction from the milder boronate affinity gel procedure in the 25 ml urine aliquot. Gherke[26] has detected a peak in the chromatograms of urine from normal volunteers and adenoid carcinoma patients which elutes with retention times similar to the standard mcm^{5}s^{2}U. The urinary nucleoside II that we have isolated and characterized may very well originate from the natural labile parent methyl ester mcm^{5}s^{2}U. The evidence, however, supporting I (ncm^{5}U) as a naturally occurring metabolite is certainly more persuasive.

These two urinary nucleosides I and II (or, the precursor of nucleoside II) are derived most probably from tRNA turnover. As with most modified nucleosides of tRNA, enzymes for reincorporation are not present, resulting in their excretion as catabolites in various physiological fluids, notably urine. With few exceptions, uridine in the first (Wobble) position of the anticodon region in all sequenced tRNA is replaced by hypermodified uridines or thiouridines substituted at the 5-carbon. It

has been demonstrated that some of the hypermodified uridines amplify the Wobble recognition while others, such as the 2-thio derivatives restrict the recognition. Adamiak[27] has reviewed the extensive studies performed to correlate molecular structure of hypermodified uridines with these and other properties. Although many of the functions of the Wobble modified uridines are not understood fully, they appear, however, to play a key role in codon-anticodon interactions.

The enzymatically controlled modification mechanism of nucleosides has been shown to be a sensitive indicator for monitoring normal cellular events[17]. Study of the excretion levels of these two hypermodified metabolites may prove useful in the investigation of the modification phenomena and its role in nucleic acid functions, cell differentiation and turnover rates. Their value as potential biological markers for disease state such as neoplasia is worth further investigation. In a preliminary study nucleoside I (ncm^5U) was found to be present in elevated levels in the blood and urine of lung carcinoma patients when compared to normal individuals[28].

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