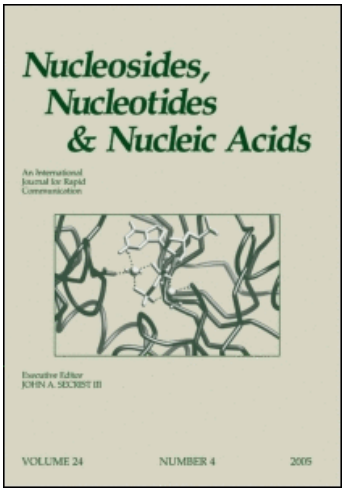

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Isolation and Characterization of 3-(3-Amino-3-Carboxypropyl)Uridine from Human Urine

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ISOLATION AND CHARACTERIZATION OF 3-(3-AMINO-3-CARBOXYPROPYL)URIDINE FROM HUMAN URINE[a]

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Abstract: A new modified nucleoside, 3-(3-amino-3-carboxypropyl)-uridine was isolated from a 24 hour collection of a normal human urine. The structure was assigned on the basis of UV, NMR and mass spectrometry data and confirmed by comparison of the spectral data and HPLC mobilities with those of an authentic sample. Origin and significance of this nucleoside in relation to tRNA is discussed. The new nucleoside is present also in the urine of cancer patients but in smaller amounts.

Introduction

We have been engaged in the isolation and characterization of modified nucleosides present in human urine[1,2]. Of more than 65 modified nucleosides known to be present in mammalian tRNA[b], 21 have been isolated from human urine. Included among these are the anticodon adja-

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a) This study was presented in part at the 35th conference of the American Society for Mass Spectrometry and Allied Topics, May 1987, Denver, CO.

b) Abbreviations used are: tRNA, transfer ribonucleic acid; acp^3U, 3-(3-amino-3-carboxypropyl)uridine; t^6A, N-[(N-9- β -D-ribofuranosylpurin-6-yl)carbamoyl]-L-threonine; TMS, trimethylsilyl; CML, chronic myelogenous leukemia; nucleoside Q, queuosine.

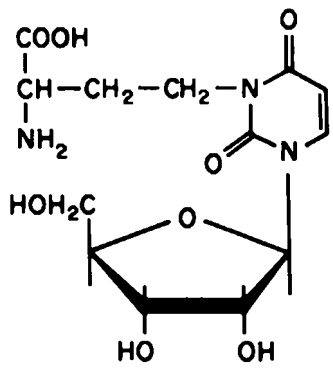

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Fig. 1. 3-(3-amino-3-carboxypropyl)uridine

cent modified nucleosides t^6A[1] and 1-methylinosine[1]. In addition to these nucleosides, a number of other modified nucleosides such as 2'-O-methyluridine[3] and N^2,N^2-dimethylguanosine[1] are also excreted routinely in human urine. With the exception of queuosine[4] and inosine[5], the modified nucleosides are biosynthesized by alteration of the four common nucleosides present within the tRNA precursor polynucleotide chains[6]. In contrast to the insertion of modified bases such as queine and hypoxanthine, modified nucleosides cannot be incorporated into the macromolecular nucleic acids. As a result, modified nucleosides once released in the process of tRNA turnover, cannot be re-utilized; they are either metabolized or excreted intact in urine[7,8]. Much remains to be learned about the process of tRNA turnover and about the metabolism of the modified nucleosides in man.

Reported herein is the isolation and characterization of a hypermodified nucleoside identified as 3-(3-amino-3-carboxypropyl)uridine (acp^3U) (Fig. 1) isolated from the urines of a normal volunteer and two cancer patients. This study constitutes the first report on the presence of acp^3U in urine.

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 while the deionized distilled water used in reversed-phase high performance liquid chromatography (RP-HPLC) was prepared in our laboratory. Silylating reagent bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane was purchased from Regis Chemical Co. An authentic sample of acp^3U was kindly provided by Professor James A. McCloskey of the University of Utah.

Ultraviolet (UV) spectrophotometry. Ultraviolet 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. Unless stated otherwise, the chemical shifts reported here are given in (δ) ppm, and are measured from internal TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-d4). The urinary unknown was lyophilized three times from 99.5% D_2O and then dissolved in 99.9% D_2O for NMR analysis.

Mass spectrometry (MS) and gas chromatography/mass spectrometry (GC/MS). Low resolution mass spectral and GC/MS studies were carried out using a Finnigan 4000 quadrupole instrument interfaced to an INCOS data system. High resolution exact mass determinations by peak matching (R = 10,000) were performed on a Varian MAT 731 instrument. Sample introduction was via the direct probe inlet with solvent reagent removal in the probe lock prior to probe insertion.

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

A fused silica capillary column (30 meter long, 3% SE-30, 25 um film thickness) was fitted 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 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 on an Altex Model 332 gradient liquid chromatograph, equipped with an Altex model 420 system controller programmer and an Altex model 153 analytical UV detector with a 8 μL flow cell set at 254 nm and with the C-R1A Altex recorder and integrator. For the preparative chromatography, a Zorbax reversed phase ODS C_{18} column (21.2 mm x 25 cm, 8 μm) fitted with a 5.0 ml injection loop was used. For the purification and coinjection studies an Ultrasphere reversed phase ODS C_{18} column (10.0 mm x 25 cm, 5 μm) fitted with a 2.0 ml injection loop was used. The following RP-HPLC solvent systems were used for coinjection studies: A) isocratic, 5% methanol in water, flow rate, 3 ml/min; B) isocratic, 0.1 M ammonium acetate buffer, pH 5.3, flow rate, 3 ml/min; C) isocratic, 0.1 M ammonium acetate buffer, pH 3.8, flow rate, 3 ml/min; D) gradient elution with 0 → 15% methanol in water in 15 min, flow rate, 3 ml/min.

Isolation of unknown urinary nucleoside I. A 24 hr urine collection from a normal male human volunteer (1490 ml) was adjusted to pH 3.6, filtered and passed through a column (3.8 x 50 cm) of charcoal-celite (50 g each). The column was washed with water (∿3 l) until the washings were chloride free. The column bound material was eluted with 2N NH_4OH in 50% aqueous ethanol (∿3 l) until the absorbance at 260 nm was below 0.20. The eluate was concentrated to 100 ml and applied to a column (3.8 x 50 cm) of AG1-X8 formate anion exchange resin (200 g) equilibrated with water. The column was washed with water (∿3 l) until the absorbance of the eluate at 260 nm decreased below 0.2. The concentrated water eluate (50 ml) was applied to a DEAE cellulose (50 g) column (3.8 x 50 cm) equilibrated with 0.14 M boric acid[10]. The column was washed with 0.14 M boric acid (1.5 l) and then eluted with 2 l of 0.7 M boric acid to obtain the desired nucleoside fraction. The 0.7 M boric acid fraction was evaporated to dryness by repeated additions of methanol (4 x 100 ml). The salt free residue was dissolved in 15 ml water, filtered and applied in three injections to a Zorbax preparative RP-HPLC column. A gradient of 0 → 25% methanol (in 1 hr) in 0.1 M ammonium acetate buffer (pH 7.0) with a flow rate of 8 ml/min was used

for elution. The unknown I was isolated from the combined pool of material eluting at 18 min. Purification to homogeneity was achieved on a semi-preparative Ultrasphere RP-HPLC column with the unknown nucleoside eluting at 26 min in water at a flow rate of 4 ml/min (160 μg).

The purified material exhibited UV spectra with a λ_{max} (nm) at 263 (pH 6.0), 263 (pH 1.0), 263 (pH 11.0) with a similar molar extinction at all pHs. This material was used for all subsequent characterization studies. The unknown nucleoside was isolated also from the 24 hr urine collections of a CML patient (42 μg) and a lung carcinoma patient (120 μg).

RESULTS

The ultraviolet spectra of the unknown urinary nucleoside I (Fig. 2a) exhibited maxima at 263 nm in neutral, acidic and alkaline media with almost no change in the molecular extinction coefficients. These maxima coupled with the similar molecular extinction coefficients at the above pHs is suggestive of an N-3 substituted uridine. Retention time of the urinary nucleoside I on RP-HPLC and differences in NMR resonances clearly excluded 3-methyluridine as a structure for this unknown. Investigation of other known modified nucleosides suggested that this substance could be the other 3-substituted uridine occurring in tRNA. This led to the comparison of the UV spectra and other physico-chemical parameters of the unknown nucleoside with those of acp^3U (Fig. 2b)[11].

The NMR spectrum of the unknown nucleoside in D_2O revealed a pair of doublets centered at 7.89 ppm and 5.97 ppm suggesting that these could be assigned to the C_6-H and C_5-H protons of the uridine moiety (Fig. 3a). A doublet centered at 5.94 ppm and multiple peaks in the 3.6-4.5 ppm region were consistent with the splitting patterns of the anomeric proton and the other ribose protons, respectively. In addition, the multiplet centered at 2.25 ppm could be assigned to the protons on the β-carbon of the side chain >CH-$\underline{CH_2}CH_2$ attached to a pyrimidine moiety. The NMR spectrum of authentic acp^3U in D_2O revealed 3 sets of doublets at 7.89, 5.98 and 5.95 ppm and a multiplet centered at 2.23 ppm (Fig. 3b, Table 1). These resonances agreed well with those present in the NMR spectrum of the urinary nucleoside I.

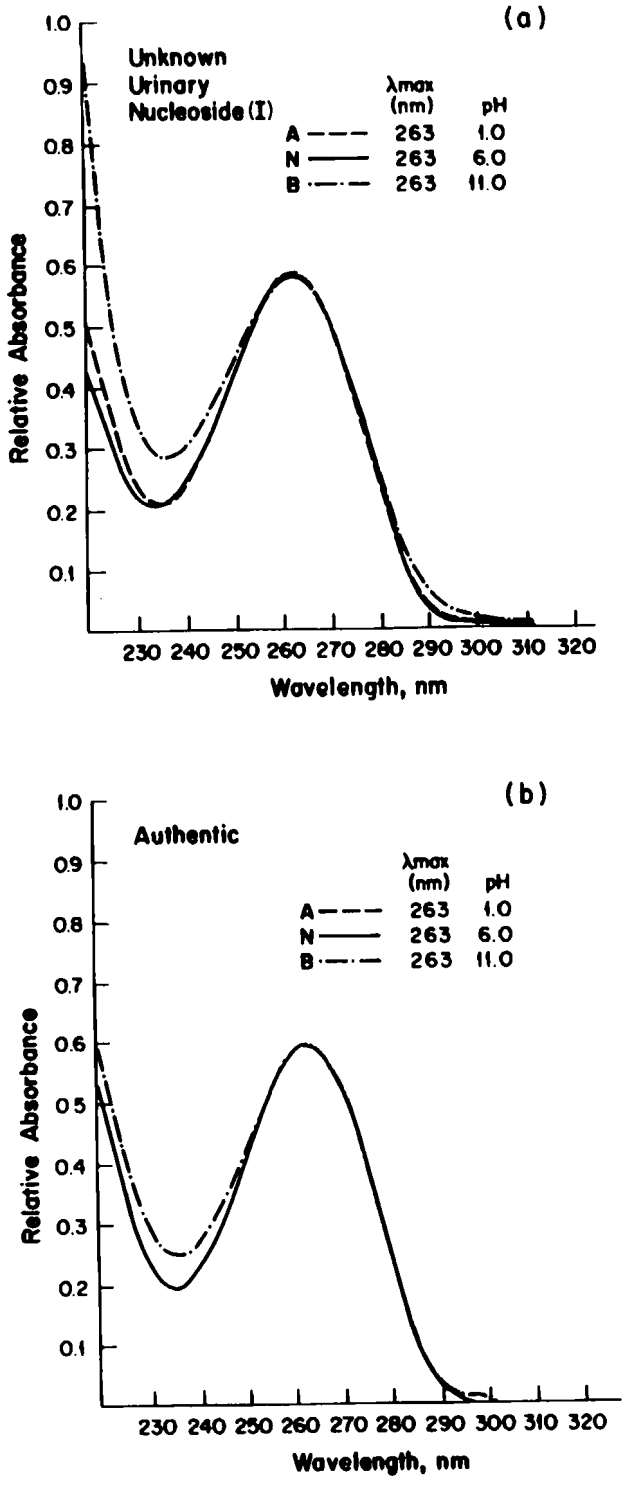

Fig. 2. Ultraviolet spectra of (a) unknown urinary nucleoside (I), and (b) authentic acp^3U.

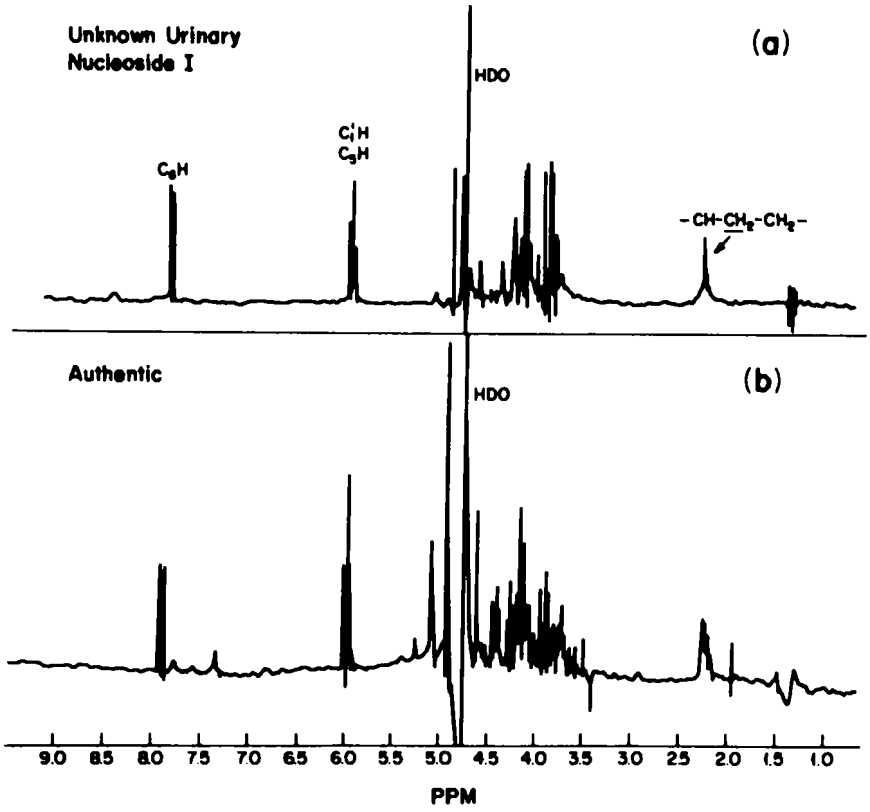

Fig. 3. NMR spectra of (a) unknown urinary nucleoside and (b) authentic acp^{3}U in D_2O.

Mass spectrometry studies gave further insight into the structure of the unknown urinary nucleoside I. The TMS derivative of urinary unknown I gave a molecular ion at m/z 633 with an M-15 ion at m/z 618 (Fig. 4a). Based on the mass spectra obtained by McCloskey and his associates[11] this is a spectrum of the tetra trimethylsilyl derivative $(TMS)_4$ of acp^{3}U. The measured exact mass of the M-15 ion was 618.2543 suggesting a molecular composition of $C_{25}H_{51}N_3O_8Si_4$ (calcd. 618.2519) for the molecular ion of the silylated compound and $C_{13}H_{19}N_3O_8$ for the free nucleoside. The ions at masses 516 and 461 are unique to this urinary nucleoside and arise from the modified base, while the ions at m/z 349, 259, 243 and 217 correspond to the unmodified sugar[11,12]. High resolution mass measurement of the ion at

Table 1

Chemical Shifts (δ) and J Values (Hz) for Unknown Urinary Nucleoside I Compared with Authentic acp^3U in D_2O

Compounds	C_6-H(J)	C_5-H(J)	C_1'-H(J)	>CH-CH_2-CH_2
Unknown urinary nucleoside (I)	7.89 (8.0)	5.97 (8.1)	5.94 (4.3)	2.25(m)
Authentic acp^3U	7.89 (8.1)	5.98 (8.0)	5.95 (4.2)	2.23(m)

m = multiplet.

516.2378 shows that it represents the characteristic loss of CO_{2TMS}[11] (calcd. 516.2381), in further support of the molecular composition of $C_{13}H_{19}N_3O_8$, corresponding to a molecular weight of 345 for underivatized I.

The mass spectra of the tetra TMS derivative of the authentic sample are identical to those of the urinary unknown I (Fig. 4b). In addition to the tetra TMS derivative, a penta TMS derivative was also formed from both the unknown I and the synthetic acp^3U. The latter derivative gave a molecular ion at m/z 705 and M-15 ion at m/z 690.

The retention times on RP-HPLC of the naturally occurring nucleoside I and that of the authentic acp^3U are very similar in four solvent systems as shown in Table 2. On coinjection with authentic acp^3U, the mixture eluted as a single discrete homogenous peak in four solvent systems further supporting the identity of the unknown. Complete agreement in UV, NMR and GC/MS spectral data and HPLC and GC retention times with those of the authentic sample unequivocally identified the unknown urinary nucleoside I as 3-(3-amino-3-carboxypropyl)-uridine.

DISCUSSION

The new nucleoside acp^3U was isolated in the amount of 160 μg from the 24 hour urine collection of a normal human volunteer. It was

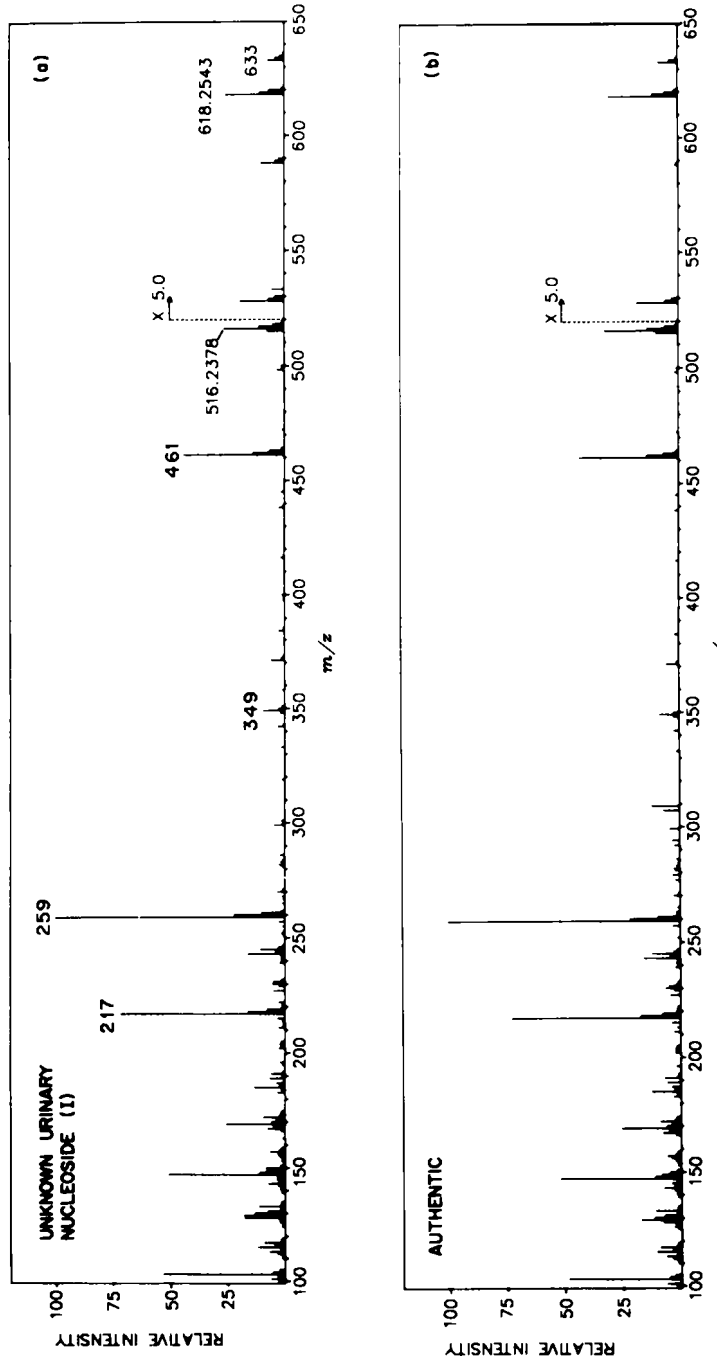

Fig. 4. Mass spectra of trimethylsilyl derivatives (TMS4) of (a) unknown urinary nucleoside I, (b) authentic acp3U.

Table 2

Comparison of RP-HPLC Retention Time (min.) of Unknown Urinary Nucleoside (I) and Authentic acp^3U

Compounds	Solvent System* (A)	(B)	(C)	(D)
Unknown urinary nucleoside (I)	9.15	19.40	11.83	11.81
Authentic acp^3U	9.07	19.74	12.06	11.76
Mixture of I and authentic acp^3U	9.28	19.36	12.01	11.76

*See Material and Methods section for solvents.

found also in the 24 hr urine collections of one CML (42 µg) and lung carcinoma (120 µg) patient. This hypermodified nucleoside was originally isolated from the extra region (loop III) of $tRNA^{Phe}$ of *E. coli*[11,13,14]. The ubiquitous presence of acp^3U in tRNA from *Drosophila melanogaster*[15], wheat germ[16], lupine seed[17], rat[18], and bovine liver as well as human liver and placenta[19] suggests its conservation in evolution. One notable contrast is its location in the extra loop (loop III) in the procaryote *E. coli* tRNA and its relocation into the dihydrouridine loop (loop I) in the higher eucaryotes. Another interesting feature is its anomalous location in tRNA, since more than 20 hypermodified nucleosides are situated either in the first position (Wobble position) of the anticodon, e.g. nucleoside Q, or adjacent to the 3'-end of the anticodon, e.g. t^6A[20]. The relevance of acp^3U in terms of location and function remains unknown. To date, detection of acp^3U has been exclusively through tRNA sequencing. Excretion of the free nucleoside I in human urine or any other biological fluids has not been reported previously.

As to the origin of this material, Nishimura[21] has shown that biosynthesis of acp^3U in procaryote tRNA occurs at the macromolecular level. The proposed mechanism involves the cleavage of S-adenosyl-L-methionine to generate 5'-deoxy-5'-methylthioadenosine and a 3-amino-3-carboxypropyl moiety which reacts with an appropriate uridine in tRNA to form acp^3U at a specific site. Similar mechanisms exist in mammalian

systems for the biosynthesis of acp^3U[22]. In analogy with the other modified nucleosides, it is suggested that the urinary acp^3U is derived from human tRNA turnover[7].

Any deficiency in substrate or modifying enzyme could result in undermodified (hypomodified) tRNA[23]. The detection of decreased levels of acp^3U in the urines of the two cancer patients may suggest a perturbation of this modification mechanism in the neoplastic state. For example, the hypermodified nucleoside Q was absent or markedly reduced in a variety of tumor tRNAs[24,25] suggesting a relationship between the neoplasia and hypomodification[26-28]. Rapidly regenerating normal rat liver was also found hypomodified for nucleoside Q and for acp^3U[29]. It would appear therefore, that growth regulation and tRNA turnover rates significantly influence the modification phenomenon.

Functions of hypermodified nucleosides in general are not well understood; however their structural complexity and specific site locations encourages one to speculate that they might play a definite role in the regulation of tRNA interactions[30,31]. While the specific significance of acp^3U remains unclear[17,32], its ubiquitous presence in a wide range of organisms invites speculation as to its importance in tRNA biomechanisms. As has been suggested, a decreased urinary excretion level may mark an increase in abnormal cellular processes occurring with the neoplastic transformation. Altered levels of modified nucleosides excreted in the urine may, therefore, prove useful for monitoring abnormal enzymatic and biochemical cellular events.

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