Structural Analysis of $O^{2'}$-Methyl-5-carbamoylmethyluridine, a Newly Discovered Constituent of Yeast Transfer RNA†

M. W. Gray‡

With the Technical Assistance of Carl Thomas

ABSTRACT: A compound tentatively identified as $O^{2'}$-methyl-5-carboxymethyluridine (cm^5Um) was recently isolated in this laboratory from bulk yeast transfer RNA (Gray, M. W. (1975), *Can. J. Biochem. 53*, 735–746). Alkaline hydrolysis of yeast tRNA releases this nucleoside as part of an alkali-stable dinucleotide, cm^5Um-Ap, from which sufficient cm^5Um was prepared in the present investigation for a detailed examination of its properties. The ultraviolet absorption spectra and chromatographic and electrophoretic properties of cm^5Um were consistent with the proposed structure, which was confirmed by characterization of the base and sugar moieties as 5-carboxymethyluracil and 2-*O*-methylribose, respectively. Snake venom hydrolysis of yeast tRNA releases cm^5Um in the form of a carboxyl-blocked 5′-nucleotide, designated pU-2. Identification of the alkali-labile blocking group in pU-2 as an amide was based on quantitative assay for ammonia released upon acid hydrolysis of the corresponding nucleoside, U-2, and by chromatographic comparison of U-2 with the semisynthetic methyl ester and amide derivatives of cm^5Um (mcm^5Um and ncm^5Um, respectively). Quantitative analysis has indicated that ncm^5Um may be confined to a single species of yeast tRNA. In view of the unique localization (the "Wobble" position of the anticodon sequence) and coding properties (pairing with A but not with G) of other cm^5U derivatives in transfer RNA, the dinucleotide cm^5Um-Ap may be derived from the first two positions of the anticodon sequence of a yeast tRNA species recognizing an NUA codon. This predicts that $O^{2'}$-methyl-5-carbamoylmethyluridine will be found in an isoleucine, leucine, or valine isoacceptor.

Modification of the "Wobble" (Crick, 1966) nucleoside in transfer RNA appears to be a mechanism for altering the codon–anticodon interaction, either by amplifying or restricting the number of codons in mRNA recognized by a particular anticodon sequence in tRNA (Nishimura, 1972, 1974; Weiss, 1973). Among the hypermodified nucleosides occupying the "Wobble" position of the anticodon sequence are derivatives of 5-carboxymethyluridine (cm^5U, **1**)[1], a carboxyl-containing nucleoside originally isolated as the 2′(3′)-nucleotide from alkaline hydrolysates of yeast and wheat embryo transfer RNA (Gray and Lane, 1967, 1968). In native tRNA, this nucleoside is present primarily (if not exclusively) in a carboxyl-blocked form (Gray and Lane, 1968), and both the methyl ester (5-carbomethoxymethyluridine, mcm^5U, **2**) (Tumaitis and Lane, 1970) and the amide (5-carbamoylmethyluridine, ncm^5U, **3**) (Dunn and Trigg, 1975) derivatives of cm^5U have been identified as constituents of tRNA. The occurrence of mcm^5U in the "Wobble" position in brewer's yeast $tRNA^{Arg}_3$ has recently been demonstrated (Kuntzel et al., 1975), and there is evidence that the same compound is present in other species of yeast tRNA (Kennedy and Lane, 1975). A sulfur analogue of mcm^5U, 2-thio-5-carbomethoxymethyluridine (s^2mcm^5U) (Baczynskyj et al., 1968; Kwong and Lane, 1970), is present in the "Wobble" position of the anticodon in two species of baker's yeast tRNA, $tRNA^{Lys}_2$ (Madison et al., 1972) and $tRNA^{Glu}_3$ (Kobayashi et al., 1974). The latter isoacceptor has been shown to respond specifically to the codon GAA, but not to GAG, in the ribosomal binding assay (Sekiya et al., 1969). The restricted coding properties of yeast $tRNA^{Glu}_3$ were originally ascribed to differences in the hydrogen bonding capacity of oxygen vs. sulfur at the C-2 position of s^2mcm^5U (Yoshida et al., 1971), but chemical modification studies (Sen, 1974) suggest that the carboxymethyl substituent, rather than the thio group, may be the principal determinant of the coding properties of s^2mcm^5U-containing tRNA species.

A compound having the properties of the $O^{2'}$-methyl derivative of cm^5U (i.e., cm^5Um, **4**) was recently identified in this laboratory as a trace constituent of bulk yeast tRNA (Gray, 1975). This derivative was released in the form of an alkali-stable dinucleotide, cm^5Um-Ap, by alkaline hydrolysis of yeast tRNA, and as a carboxyl-blocked 5′-nucleotide (designated pU-2) by snake venom hydrolysis of the same RNA. The present investigation was undertaken in order to characterize more definitively the putative $O^{2'}$-methyl-5-carboxymethyluridine, and to determine the nature of the blocking group in pU-2.

Materials and Methods

Materials

Uridine (U) and $O^{2'}$-methyluridine (Um) were commercial products (Calbiochem and ICN Pharmaceuticals, respectively). 5-Carboxymethyluridine (from yeast transfer RNA) and 5-carboxymethyluracil (cm^5u) were prepared by published methods (Gray and Lane, 1968). The amide and methyl ester derivatives of cm^5U, synthesized by the procedures of Fissekis and Sweet (1970), were kindly provided by Dr. J. D. Fissekis, Sloan-Kettering Institute for Cancer Research. Brewer's yeast transfer RNA was purchased from Boehringer Mannheim. Analysis by polyacrylamide gel electrophoresis (Gray, 1974b) indicated that this material contained greater than 85% tRNA, and that DNA and high-molecular-weight rRNA were absent. Other commercial products were *Vipera russelli* venom (Ross

† From the Department of Biochemistry, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia B3H 4H7. *Received January 14, 1976.* Supported by a grant (MA-4124) from the Medical Research Council of Canada.

‡ Medical Research Council (Canada) Scholar.

[1] The structures and abbreviated nomenclature of the various 5-carboxymethyluridine derivatives discussed in this report are presented in Figure 1. Abbreviation used: DEAE, diethylaminoethyl.

Allen Reptile Institute, Silver Springs, Fla.), *Escherichia coli* alkaline phosphatase (Worthington Biochemicals or Sigma Chemical Co.), and DEAE-cellulose (No. 70; Schleicher & Schuell, Inc., Keene, N.H.).

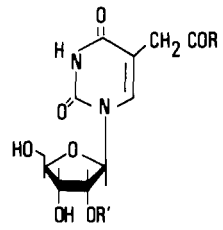
Methods

Chromatographic and Electrophoretic Techniques. Except in the case of system B, descending paper chromatography was conducted in tanks preequilibrated with developing solvent. Solvents (v/v) were: A and A′, 95% ethanol–water, 4:1, B, saturated ammonium sulfate–2-propanol, 40:1; C, 95% ethanol–1 M ammonium acetate, 7:3, saturated with boric acid and adjusted to pH 9 with ammonium hydroxide; D, 1-butanol–95% ethanol–water, 50:18:15; E, upper phase of ethyl acetate–water–1-propanol, 4:2:1; F, upper phase of ethyl acetate–16% formic acid–2-ethoxyethanol, 4:2:1. Untreated Whatman No. 1 chromatography paper (systems A′ and C–F) or ammonium sulfate impregnated Whatman No. 1 paper (systems A and B; Singh and Lane, 1964) was used. Paper electrophoresis was carried out on Whatman No. 1 paper in a Durrum-type electrophoresis apparatus (Beckman) at 500 V for 1 h. Electrophoresis buffers were: (1) 1 M formic acid (pH 1.8), (2) 0.025 M ammonium acetate (pH 3.5), (3) 0.025 M ammonium acetate (pH 5.0), (4) 0.025 M ammonium formate (pH 9.2), (5) 0.025 M sodium tetraborate (pH 9.2).

Isolation of the Dinucleoside Monophosphate, cm^{5}Um-A, from Alkaline Hydrolysates of Yeast Transfer RNA and Preparation of the Nucleoside, cm^{5}Um. Alkaline hydrolysis of yeast tRNA (2 g) and fractionation of the hydrolysis products (on a 4.5 × 24 cm column of DEAE-cellulose) were carried out by the procedures of Lane (1965). The post-dinucleotide fraction (containing primarily nucleoside 2′(3′),5′-diphosphates, pNp) was treated with alkaline phosphatase (Gray, 1975). The resulting hydrolysate was diluted to 200 ml with 0.025 M Tris-formate (pH 7.8) and passed into a 2.5 × 5 cm column of DEAE-cellulose (formate) equilibrated with the same buffer. The column was washed with the 0.025 M buffer until elution of nucleosides was complete. After removal of residual buffer with a water wash, cm^{5}Um-A (resulting from dephosphorylation of cm^{5}Um-Ap present initially in the post-dinucleotide fraction) was eluted with 1 M formic acid (pH 1.8). Fractions containing uv-absorbing material (which appeared immediately after the column void volume) were combined and taken to dryness in vacuo, after adjustment of the pH to 4.5 with pyridine. The residue was reevaporated from dilute ammonium hydroxide, after which the final salt-free material was chromatographed in system A. The prominent band of cm^{5}Um-A (R_f 0.48) was attached to a fresh section of ammonium sulfate impregnated Whatman No. 1 paper, further purified by chromatography in system B, and recovered by charcoal desalting (Gray and Lane, 1967).

To prepare cm^{5}Um, cm^{5}Um-A was treated with a mixture of purified snake venom phosphodiesterase and alkaline phosphatase (Gray, 1974a), and the products were separated by electrophoresis in system 1. At this pH (1.8), cm^{5}Um remained close to the origin, while A migrated toward the cathode. The cm^{5}Um recovered by this procedure was chromatographically and electrophoretically homogeneous.

Isolation of the Nucleotide, pU-2, from Snake Venom Hydrolysates of Yeast tRNA and Preparation of the Nucleosides, U-2 and U-2.* Venom hydrolysis of yeast tRNA (2 g) and fractionation of the hydrolysis products (on a 4.5 × 15 cm column of DEAE-cellulose) were carried out as previously described (Gray, 1975). The mononucleotide fraction (containing $O^{2'}$-methylnucleoside 5′-monophosphates, pNm) was subfractionated on a 2.5 × 30 cm column of DEAE-cellulose (formate) in the presence of 1 M formic acid. Fractions containing pUm as the major component (Subfraction M-4; Gray, 1974b) were combined and the nucleotides recovered by flash evaporation, as above. The components of subfraction M-4 were resolved by chromatography in system A′ into three uv-absorbing bands, containing (in order of increasing mobility): band 1, unknown pN-1 (Gray, 1975), which has been identified as 2-amino-4-oxo-5-*N*-methylformamido-6-(5′-phosphoribosyl)aminopyrimidine (an alkaline conversion product of 7-methylguanosine 5′-phosphate, arising during the venom hydrolysis step); band 2, pU-2 plus unknown pN-1*, the latter identified as 2,6-diamino-4-oxo-5-*N*-methylformamidopyrimidine and resulting from hydrolysis of the *N*-glycosyl bond in pN-1 during the recovery and storage of pNm Subfraction M-4); band 3, pUm. Electrophoresis of the material in band 2 in 1 M formic acid separated pU-2 (which migrated as an anion) from pN-1* (which migrated as a cation).



1	R=OH,	R′=H	(5-carboxymethyluridine, cm^{5}U)
2	R=OCH_3,	R′=H	(5-carbomethoxymethyluridine, mcm^{5}U)
3	R=NH_2,	R′=H	(5-carbamoylmethyluridine, ncm^{5}U)
4	R=OH,	R′=CH_3	($O^{2'}$-methyl-5-carboxymethyluridine, cm^{5}Um)
5	R=OCH_3,	R′=CH_3	($O^{2'}$-methyl-5-carbomethoxymethyluridine, mcm^{5}Um)
6	R=NH_2,	R′=CH_3	($O^{2'}$-methyl-5-carbamoylmethyluridine, ncm^{5}Um)

FIGURE 1: Structural formulas of 5-carboxymethyluridine derivatives.

To prepare U-2, pU-2 was treated with alkaline phosphatase. To prepare U-2* (the alkaline conversion product of U-2), pU-2 was first incubated in 1 M NaOH at room temperature for 90 h, to yield pU-2*, which was recovered by adsorption to and elution from DEAE-cellulose (Lane, 1965). Treatment of pU-2* with alkaline phosphatase gave U-2*. Both U-2 and U-2* were recovered by electrophoresis in 1 M formic acid, and both were homogeneous in all chromatographic and electrophoretic systems tested.

Synthesis of the Methyl Ester and Amide Derivatives of $O^{2'}$-Methyl-5-carboxymethyluridine. Starting with purified cm^{5}Um, $O^{2'}$-methyl-5-carbomethoxymethyluridine (mcm^{5}Um, **5**) and $O^{2'}$-methyl-5-carbamoylmethyluridine (ncm^{5}Um, **6**) were synthesized by procedures that have been used for the preparation of the methyl ester (Tumaitis and Lane, 1970) and amide (Fissekis and Sweet, 1970) derivatives of cm^{5}U.

Analysis of the Sugar and Base Components of $O^{2'}$-Methyl-5-carboxymethyluridine. The sugar moiety of cm^{5}Um was released by hydrazinolysis (Baron and Brown, 1955), as described by Nichols and Lane (1968). As controls, commercial samples of uridine and $O^{2'}$-methyluridine and a sample of cm^{5}U from yeast tRNA were submitted to the same procedure. After chromatography in system A, the sugar components were visualized by reaction with *m*-phenylenediamine hydrochloride in 76% ethanol (Chargaff et al., 1949). The base constituent of cm^{5}Um was released by acid hydrolysis (Furukawa et al., 1965).

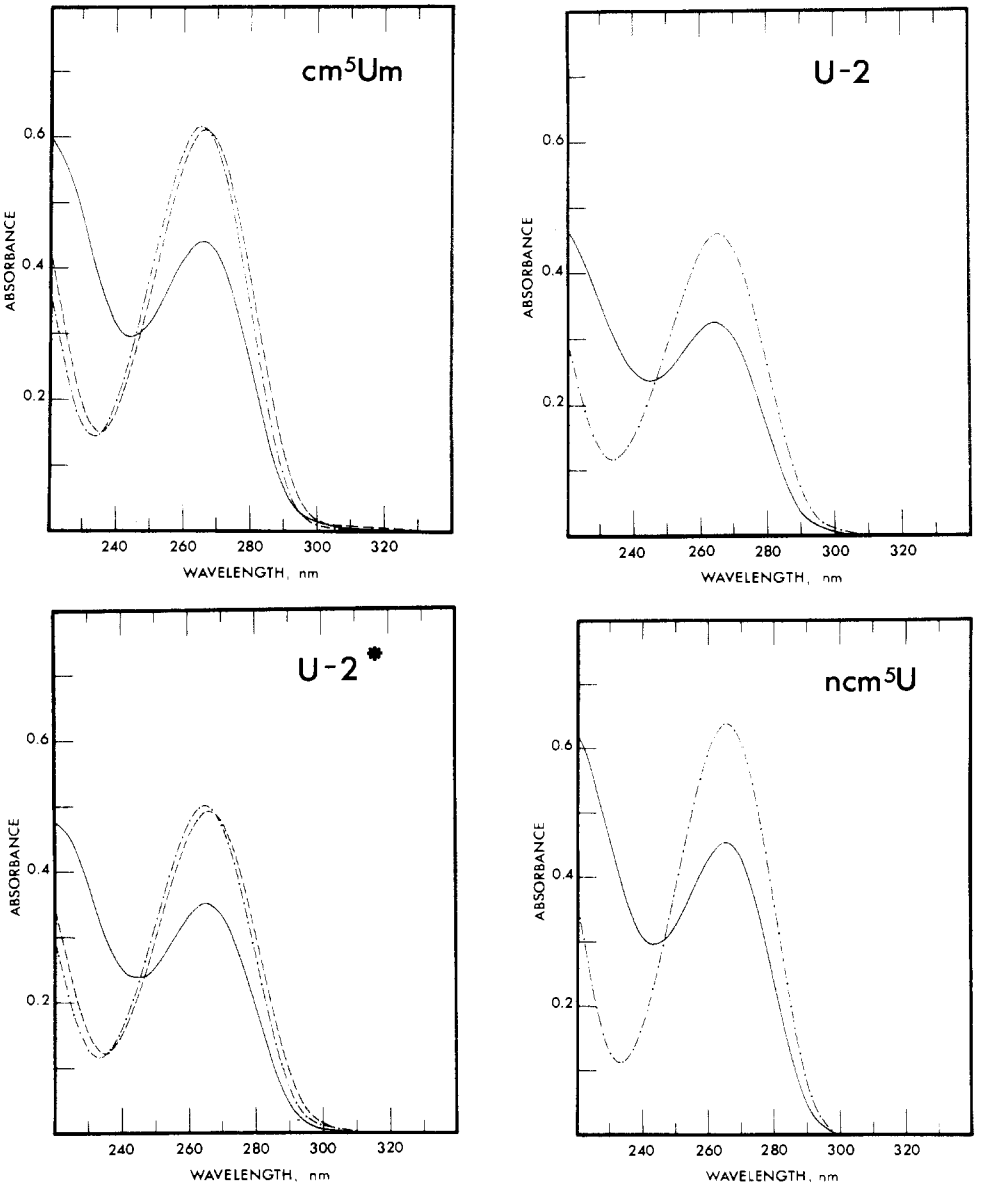


FIGURE 2: Ultraviolet absorption spectra of 5-carboxymethyluridine derivatives. Spectra were recorded on a Bausch and Lomb Spectronic 505 spectrophotometer. Compounds were first chromatographed in system A′ and eluted in water, along with appropriate blanks. Neutral spectra (- - - -) were determined on 1 ml of aqueous eluate, recorded against 1 ml of blank. Acidic spectra (-·-·-·) were recorded after addition of 10 μl of concentrated HCl to the sample and blank, and alkaline spectra (—) after a further addition of 20 μl of 10 M NaOH to the sample and blank. The acidic and neutral spectra were identical in the case of U-2 and ncm^{5}U, and only the acidic spectrum of each is presented. The values for λ_{max} (nm) (acidic, neutral, alkaline) and λ_{min} (nm) (acidic, neutral, alkaline) are: cm^{5}Um (A) 265, 266, 266; 233.5, 235, 244; U-2 (B) 265, 265, 264.5; 233, 233, 245; U-2* (C) 265, 266.5, 265; 234, 235, 245; ncm^{5}U (D) 265, 265, 265; 233, 233, 244.

Ammonia Assay. Samples of ncm^{5}U, mcm^{5}U, and U-2 were chromatographed in system A′ on Whatman No. 1 paper prewashed with water. Compounds were eluted in deionized water and the concentration of each nucleoside was determined spectrophotometrically. Aliquots containing about 0.1 μmol of nucleoside were lyophilized and submitted to acid hydrolysis (6 M HCl, 100 °C, 6 h, in sealed evacuated tubes). The ammonia produced in each hydrolysate was quantitatively assayed on a Beckman amino acid analyzer.

Results

Structural Analysis of $O^{2'}$-Methyl-5-carboxymethyluridine. The ultraviolet absorption spectra of cm^{5}Um (Figure 2A) were essentially identical with those of cm^{5}U (Gray and Lane, 1968). In particular, both cm^{5}U and cm^{5}Um displayed the slight but distinctive spectral shift, which is associated with the ionization of the carboxyl function (Kwong and Lane, 1970) between acidic and neutral pH values. The electrophoretic mobilities of cm^{5}U and cm^{5}Um were identical at five pH values, and the acquisition of a negative charge by both compounds between pH 1.8 and 9.2 was clearly evident (Figure 3).

On the other hand, cm^{5}Um was readily distinguishable from cm^{5}U by its chromatographic mobility in a number of systems (Tables I and II). The increased mobility of cm^{5}Um, relative to cm^{5}U, in nonpolar solvent systems (A, A′, D–G) and decreased mobility in a polar system (B) were consistent with the presence of an additional nonpolar group in cm^{5}Um (compare the mobilities of U and Um, Table I). The failure of cm^{5}Um to complex with borate during electrophoresis (Table III) indicated the absence of a *cis*-diol grouping in this nucleoside, as did the accelerated mobility of cm^{5}Um, relative to cm^{5}U, in system C (Table I). Coupled with the fact that cm^{5}Um was originally isolated as part of an alkali-stable dinucleotide, this suggested the presence of an $O^{2'}$-methyl group in this nucle-

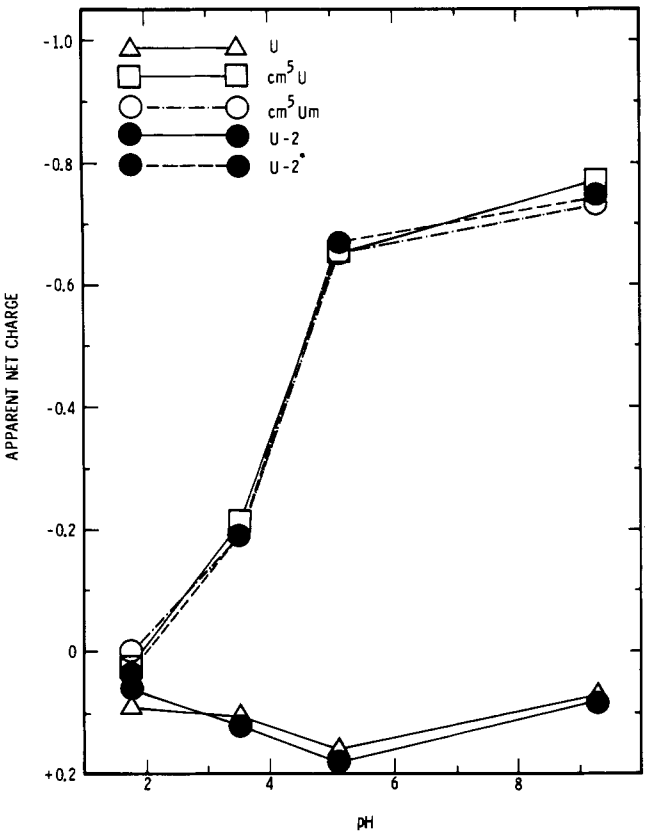


FIGURE 3: Electrophoretic mobilities of 5-carboxymethyluridine derivatives.

TABLE I: Chromatographic Identity of $O^{2'}$-Methyl-5-carboxymethyluridine (cm^5Um) and Unknown U-2*.

Compound	$R_{uridine}$ in System A	$R_{uridine}$ in System A′	$R_{uridine}$ in System B	R_f System C
U	(1.00)	(1.00)	(1.00)	0.45
Um	1.22	1.18	0.87	0.86
cm^5U	0.99	0.56	1.10	0.28
cm^5Um	1.23	0.72	0.94	0.71
U-2*	1.22	0.71	0.93	0.71
U-2	1.03	0.98	0.89	0.80

oside. Characterization of the sugar portion of cm^5Um as 2-*O*-methylribose (Table IV) provided direct evidence of $O^{2'}$-methylation.

The ultraviolet absorption spectra (λ_{max} (nm) 262 (pH 1), 264 (pH 7), and 289 (pH 13); λ_{min} 232 (pH 1), 234 (pH 7), 245 (pH 13)) of the base liberated by acid hydrolysis of cm^5Um were the same as those of synthetic 5-carboxymethyluracil (Gray and Lane, 1968). The two compounds were also chromatographically and electrophoretically identical (Table V).

Characterization of Unknown Nucleoside U-2 as a Carboxyl-Blocked Derivative of $O^{2'}$-Methyl-5-carboxymethyluridine. The ultraviolet absorption spectra of U-2 (Figure 2B) indicated that it was a 5-substituted uridine derivative, but the acidic to neutral spectral transition shown by cm^5U and cm^5Um (see Figure 2A), and diagnostic of carboxyl ionization, was absent. Lack of an ionizable carboxyl group was verified by electrophoretic analysis between pH 1.8 and 9.2 (Figure 3). Like cm^5Um, U-2 did not complex with borate during electrophoresis (Table III), indicating the absence of a *cis*-diol grouping. U-2 could be distinguished from cm^5Um by paper chromatography in a number of solvents (Tables I and II).

After alkaline treatment of U-2, the product (U-2*) was found to have properties identical to those of cm^5Um. These included ultraviolet absorption spectra (Figure 2C), chromatographic mobility (Table I), and electrophoretic behavior (Figure 3). The latter properties demonstrate the acquisition of an acidic function after alkaline treatment of U-2, as does the change in the ultraviolet absorption spectra of U-2* (Figure 2C) compared with those of U-2 (Figure 2B).

TABLE II: Chromatographic Identity of Unknown U-2 and Semisynthetic $O^{2'}$-Methyl-5-carbamoylmethyluridine (ncm^5Um).

Compound	R_f in System A	A′	D	E	F
mcm^5U	0.72	0.67	0.42	0.34	0.54
cm^5U	0.59	0.36	0.02	0.01	0.35
ncm^5U	0.43	0.38	0.14	0.04	0.20
cm^5Um	0.75	0.45	0.06	0.02	0.51
U-2	0.63	0.58	0.25	0.12	0.36
ncm^5Um	0.64	0.56	0.25	0.11	0.35
mcm^5Um	0.84	0.76	0.59	0.57	0.67

TABLE III: Electrophoretic Mobilities of 5-Carboxymethyluridine Derivatives in the Presence and Absence of Borate Ion.[a]

Compound	$R_{picrate}$[b] − Borate (System 4)	$R_{picrate}$[b] + Borate (System 5)
U	−0.09	−0.70
Um	−0.14	−0.20
cm^5U	−0.79	−1.09
cm^5Um	−0.73	−0.71
U-2*	−0.74	−0.76
U-2	−0.04	−0.20

[a] It should be noted that some of the relative mobilities listed above (and in Table V) differ quantitatively from previously published mobilities for the same compounds (Gray and Lane, 1968). At the present time, there is no obvious explanation for these differences, although the possibility that they are due to slight modifications in the electrophoretic technique (Gray, 1976) is being investigated. [b] The picrate marker was assigned a mobility of −1.00 in both systems (the minus sign indicating migration toward the anode).

TABLE IV: Characterization of the Sugar Component of Various Uridine Derivatives.

Compound	Product of Reaction with *m*-Phenylenediamine[a] $R_{uridine}$	Color
D-ribose	0.91	Orange
Hydrazine-treated		
U	0.91	Orange
cm^5U	0.92	Orange
Um	1.14	Pink
cm^5Um	1.14	Pink

[a] 2-Deoxy-D-ribose migrates slightly behind 2-*O*-methylribose in this system and gives a yellow color on reaction with *m*-phenylenediamine.

TABLE V: Electrophoretic and Chromatographic Properties of the Base Constituent of $O^{2'}$-Methyl-5-carboxymethyluridine and of Synthetic 5-Carboxymethyluracil.[a]

Compound	Electrophoretic Mobility ($R_{picrate}$) at pH				Chromatographic Mobility (R_f) in System				
	1.8	3.5	5.0	9.2	A	B	D	E	F
Natural cm^5U	+0.09	−0.27	−1.03	−1.26	0.62	0.56	0.03	0.00	0.51
Synthetic cm^5U	+0.13	−0.28	−1.00	−1.31	0.62	0.58	0.02	0.00	0.50

[a] The picrate marker was assigned an electrophoretic mobility of −1.00 at all pH values (the minus sign indicating migration toward the anode).

TABLE VI: Assay for Ammonia Liberated upon Acid Hydrolysis of 5-Carboxymethyluridine Derivatives

Nucleoside	μmol Hydrolyzed[a]	μmol NH_3 Produced	Molar Ratio, NH_3: Nucleoside
mcm^5U	0.110, 0.120	0.024, 0.033	0.22, 0.27
ncm^5U	0.0996, 0.101	0.096, 0.089	0.96, 0.88
U-2	0.101	0.097	0.96
U-2, blank[b]		0.026	-

[a] Molar extinction coefficients of 9300 for ncm^5U and U-2 (260 nm, neutral pH) were calculated from published data (Fissekis and Sweet, 1970) and from ultraviolet absorption spectra recorded in this laboratory. An extinction coefficient of 9300 was also assumed for mcm^5U, although the data of Fissekis and Sweet (1970) suggest a value of 4900 for this compound. [b] A blank area of the U-2 chromatogram was eluted with deionized water and an appropriate aliquot was lyophilized and subjected to acid hydrolysis and ammonia assay.

Nature of the Blocking Group in U-2. Because both the methyl ester (Tumaitis and Lane, 1970) and amide (Dunn and Trigg, 1975) derivatives of cm^5U had been isolated from yeast tRNA, it was assumed that unknown U-2 would prove to be either the methyl ester or amide derivative of cm^5Um. The ultraviolet absorption spectra of U-2 (Figure 2B) were essentially identical with those of ncm^5U (Figure 2D), but since the uv spectra of mcm^5U and ncm^5U are virtually indistinguishable, it was not possible on this basis alone to decide the nature of the blocking group in U-2.

As shown by the data in Table II, the relative chromatographic mobilities of U-2 and cm^5Um strongly suggested that U-2 was the amide rather than the methyl ester of cm^5Um (compare the relative mobilities of cm^5U, mcm^5U, and ncm^5U). This was confirmed by chromatographic comparison of U-2 with the semisynthetic methyl ester and amide derivatives of cm^5Um. The unknown migrated identically with ncm^5Um, but quite differently from mcm^5Um, in all systems tested (Table II). As expected, the $O^{2'}$-methyl derivatives had higher R_f values than their unmethylated counterparts in all of the (nonpolar) solvent systems listed in Table II.

Direct evidence for the presence of an amide group in U-2 was provided by analysis for ammonia liberated by acid hydrolysis of the unknown. As shown by the data in Table VI, the yield of ammonia from synthetic ncm^5U and unknown U-2 was identical, and much greater than the amount produced from synthetic mcm^5U. In the latter case, the ammonia yield was comparable to the yield from an appropriate blank containing no nucleoside, and, therefore, must represent a background level from the chromatogram. Correcting for this blank value, about 0.7 mol of ammonia was liberated per mole of U-2, acceptably close to the expected 1:1 molar ratio.

These observations establish the identity of unknown U-2 as $O^{2'}$-methyl-5-carbamoylmethyluridine.

Discussion

The development of a method (Gray, 1975) for the large-scale group isolation of the $O^{2'}$-methylnucleoside constituents of an RNA sample has allowed the detection of $O^{2'}$-methyl-5-carbamoylmethyluridine (ncm^5Um), a trace constituent of bulk yeast tRNA, and the preparation of this compound in quantities sufficient for detailed structural characterization. Quantitative assay has suggested that ncm^5Um may be confined to a single isoaccepting species of tRNA in yeast (Gray, 1975), and the isolation of the dinucleotide cm^5Um-Ap from yeast tRNA allows one to predict which tRNA species might contain ncm^5Um. Since derivatives of cm^5U have to date only been found in the "Wobble" position of the anticodon sequence, it is likely that cm^5Um-Ap is derived from the first two positions of the anticodon sequence. If this is the case, and if ncm^5Um pairs with A but not with G, then the tRNA species containing ncm^5Um should recognize an NUA codon, and should, therefore, be an isoleucine (AUA), valine (GUA), or leucine (CUA, UUA) isoacceptor. A 5-substituted uridine, which may well be a cm^5U derivative, has been detected in the "Wobble" position of the anticodon sequence of yeast $tRNA^{Val}_{2a}$ (Axel'rod et al., 1974), although the fact that this component was liberated as a mononucleotide during pancreatic ribonuclease digestion eliminates the possibility that it could be the $O^{2'}$-methyl derivative of cm^5U described here.

If sugar methylation is an event secondary to the addition of the carbamoylmethyl side chain, then formation of ncm^5Um may be viewed as involving the selective $O^{2'}$-methylation of about 20% of the total ncm^5U residues in yeast tRNA (the latter nucleoside being present at a level of ca. 0.1 mol % in yeast tRNA (Dunn and Trigg, 1973, 1975)). It remains to be determined what factor(s) restrict the additional $O^{2'}$-methylation to a limited proportion of the tRNA species containing ncm^5U, and whether there are functional differences between ncm^5U and ncm^5Um at the molecular level. Notably, $O^{2'}$-methyl-5-carbamoylmethyluridine 5'-phosphate has not been detected in venom hydrolysates of wheat embryo tRNA (Gray, 1975), even though the latter RNA apparently contains only the amide derivative of cm^5U (Dunn and Trigg, 1973, 1975). If the absence of ncm^5Um in wheat embryo tRNA reflects the absence of the necessary $O^{2'}$-methyltransferase, and if the site that is normally $O^{2'}$-methylated in yeast tRNA is available in wheat embryo tRNA, then a heterologous system comprising wheat embryo tRNA and yeast enzymes may prove useful in studies of the biosynthesis of ncm^5Um.

The esterified methyl groups of the carbomethoxymethyl

side chains of mcm^5U and s^2cm^5U in yeast tRNA are known to be derived from *S*-adenosylmethionine both in vivo (Tumaitis and Lane, 1970; Kwong and Lane, 1970) and in vitro (Bronskill et al., 1972; Kennedy and Lane, 1975; Kuntzel et al., 1975). It is not yet known whether there exists a comparable enzyme system capable of carrying out the amidation of the carboxyl function of cm^5U residues in tRNA, and which, therefore, might be involved in the biosynthesis of ncm^5U and ncm^5Um. It has been suggested (Dunn and Trigg, 1975) that mcm^5U may be an intermediate in the biosynthesis of ncm^5U, an hypothesis that could explain why dormant wheat embryos contain an enzyme activity capable of generating mcm^5U (a component not found in the tRNA of dormant embryos) in saponified yeast tRNA (Bronskill et al., 1972). If mcm^5U is indeed a biosynthetic precursor of ncm^5U; then the relative proportion of the two derivatives in tRNA could be a reflection of the physiological state of an organism. Some support for this idea is provided by recent analytical data (Dunn and Trigg, 1975) that indicates that although the total proportion of cm^5U in different samples of yeast tRNA is fairly constant, the relative proportions of amide and methyl ester can vary considerably in different batches. In this context, it will be of interest to look for mcm^5U in the tRNA of *germinating* wheat embryos.

The existence of two types of carboxyl blocking for the cm^5U residues of tRNA raises the question of whether the biological activity of cm^5U-containing tRNAs might be dependent on the nature of the carboxyl-blocking group. Enzymatic esterification and deesterification of the carbomethoxymethyl group in intact tRNA has been proposed as a possible mechanism for altering the properties of a tRNA molecule (Baczynskyj et al., 1968; Kennedy and Lane, 1975). Likewise, modulation of the biological activity of a cm^5U-containing tRNA species might be effected by an enzyme-catalyzed transition from one carboxyl-blocked derivative to another. While any such regulatory roles for cm^5U derivatives in tRNA remain to be demonstrated, a search for modification and demodification enzymes would seem to be warranted, particularly in view of the recent demonstration of an enzyme activity that specifically removes the isopentenyl side chain from isopentenylated species of tRNA (McLennan, 1975). Further studies of the structure of cm^5U derivatives in tRNA, of their localization in purified isoacceptors, and of enzymes involved in their biosynthesis and subsequent metabolism should help to clarify the biological role(s) of these interesting modified nucleosides.

Acknowledgments

This investigation was greatly facilitated by the provision of generous gifts of ncm^5U and mcm^5U by Dr. J. D. Fissekis, Sloan-Kettering Institute for Cancer Research. I am also indebted to Dr. J. A. Verpoorte and R. Breckon of this department for their generous assistance with the ammonia assays.

References

Axel'rod, V. D., Kryukov, V. M., Isaenko, S. N., and Bayev, A. A. (1974), *FEBS Lett. 45,* 333-336.

Baczynskyj, K., Biemann, K., and Hall, R. H. (1968), *Science 159,* 1481-1483.

Baron, F., and Brown, D. M. (1955), *J. Chem. Soc.* 2855-2860.

Bronskill, P., Kennedy, T. D., and Lane, B. G. (1972), *Biochim. Biophys. Acta 262,* 275-282.

Chargaff, E., Vischer, E., Doniger, R., Green, C., and Misani, F. (1949), *J. Biol. Chem. 177,* 405-416.

Crick, F. H. C. (1966), *J. Mol. Biol. 19,* 548-555.

Dunn, D. B., and Trigg, M. D. M. (1973), *John Innes Inst. Annu. Rep. 64,* 147-150.

Dunn, D. B., and Trigg, M. D. M. (1975), *Biochem. Soc. Trans. 3,* 656-659.

Fissekis, J. D., and Sweet, F. (1970), *Biochemistry 9,* 3136-3142.

Furukawa, Y., Kobayashi, K., Kanai, Y., and Honjo, M. (1965), *Chem. Pharm. Bull. 13,* 1273-1278.

Gray, M. W. (1974a), *Biochim. Biophys. Acta 374,* 253-257.

Gray, M. W. (1974b), *Biochemistry 13,* 5453-5463.

Gray, M. W. (1975), *Can. J. Biochem. 53,* 735-746.

Gray, M. W. (1976), *Can. J. Biochem. 54,* 413-422.

Gray, M. W., and Lane, B. G. (1967), *Biochim. Biophys. Acta 134,* 243-257.

Gray, M. W., and Lane, B. G. (1968), *Biochemistry 7,* 3441-3453.

Kennedy, T. D., and Lane, B. G. (1975), *Can. J. Biochem. 53,* 1-10.

Kobayashi, T., Irie, T., Yoshida, M., Takeishi, K., and Ukita, T. (1974), *Biochim. Biophys. Acta 366,* 168-181.

Kuntzel, B., Weissenbach, J., Wolff, R. E., Tumaitis-Kennedy, T. D., Lane, B. G., and Dirheimer, G. (1975), *Biochimie 57,* 61-70.

Kwong, T. C., and Lane, B. G. (1970), *Biochim. Biophys. Acta 224,* 405-412.

Lane, B. G. (1965), *Biochemistry 4,* 212-219.

Madison, J. T., Boguslawski, S. J., and Teetor, G. H. (1972), *Science 176,* 687-689.

McLennan, B. D. (1975), *Biochem. Biophys. Res. Commun. 65,* 345-351.

Nichols, J. L., and Lane, B. G. (1968), *Biochim. Biophys. Acta 166,* 605-615.

Nishimura, S. (1972), *Prog. Nucleic Acid Res. Mol. Biol. 12,* 49-85.

Nishimura, S. (1974), *MTP Int. Rev. Sci.: Biochem., Ser. One 6,* 289-322.

Sekiya, T., Takeishi, K., and Ukita, T. (1969), *Biochim. Biophys. Acta 182,* 411-426.

Sen, G. C. (1974), Abstracts of the Seventeenth Annual Meeting of the Canadian Federation of Biological Societies, p 88.

Singh, H., and Lane, B. G. (1964), *Can. J. Biochem. 42,* 87-93.

Tumaitis, T. D., and Lane, B. G. (1970), *Biochim. Biophys. Acta 224,* 391-403.

Weiss, G. B. (1973), *J. Mol. Evol. 2,* 199-204.

Yoshida, M., Takeishi, K., and Ukita, T. (1971), *Biochim. Biophys. Acta 228,* 153-166.