5-Carboxymethyluridine, a Novel Nucleoside Derived from Yeast and Wheat Embryo Transfer Ribonucleates*

M. W. Gray† and B. G. Lane‡

ABSTRACT: A carboxyl-containing nucleoside, 5-carboxymethyluridine, has been isolated from the transfer ribonucleates of bakers' yeast, brewers' yeast, and wheat embryo. The 2′- and 3′-nucleotides of 5-carboxymethyluridine were isolated from alkali hydrolysates of the purified transfer ribonucleate preparations. A phosphodiesterase hydrolysate of wheat embryo transfer ribonucleates was examined for the presence of 5-carboxymethyluridine 5′-phosphate, but none could be detected. However, after alkali treatment of the 5′-nucleotides produced by the action of phosphodiesterase on wheat embryo transfer ribonucleates, a compound having the properties of the 5′-nucleotide of 5-carboxymethyluridine was isolated. This observation suggests that 5-carboxymethyluridine does not occur as the free acid in native transfer ribonucleates, but that before alkali hydrolysis the carboxyl function is substituted by an alkali-labile group. One possibility is that 5-carboxymethyluridine may occur as a saponifiable carboxymethyl ester in native transfer ribonucleates. The base derived from 5-carboxymethyluridine was identical with synthetic 5-carboxymethyluracil, and distinctly different from 6-carboxymethyluracil, in its spectral, electrophoretic, and chromatographic properties. 5-Carboxymethyluridine 2′(3′)-phosphate accounts for 0.34 and 0.15 mole % of the products formed by alkali hydrolysis of transfer ribonucleates from brewers' yeast and wheat embryo, respectively.

Alkali hydrolysis of tRNA or rRNA releases nucleosides (N) and nucleoside 2′(3′),5′-bisphosphates (pNp) from chain termini, and nucleoside 2′(3′)-monophosphates (Np) and alkali-stable dinucleotides (NmpNp)[1] from internal positions of the ribonucleate chains.[2] By ion-exchange chromatography on DEAE-cellulose columns, using urea-containing eluents (Tomlinson and Tener, 1963), these four types of product can be resolved from one another according to their different net charges at pH 7.8 (N, 0; Np, −2; NmpNp, −3; and pNp, −4). Stepwise elution with urea-containing eluents of increasing ionic strength cleanly resolves the three *minor* fractions (N, NmpNp, pNp) from one another, and from the *major* fraction (Np), which contains the bulk (>90%) of the hydrolysis products. The individual compounds in each of these fractions can then be resolved by column and/or paper chromatographic procedures (Singh and Lane, 1964b; Lane, 1965; Hudson *et al.*, 1965).

In the case of $O^{2'}$-methylated dinucleotides (NmpNp), it is convenient to effect a preliminary separation by chromatography of the bulk dinucleotides on DEAE-cellulose at pH 1.8, and then, following removal of phosphomonoester groups, to effect a final separation of the resulting dinucleoside phosphates (NmpN) by two-dimensional paper chromatography (Gray and Lane, 1967). In the course of examining the dinucleotides from wheat embryo tRNA (Hudson *et al.*, 1965) and yeast tRNA (Gray and Lane, 1967), an unusual compound was detected on two-dimensional paper chromatograms of dinucleoside phosphates ("T?," Figure 3, Gray and Lane, 1967). This unusual compound was spectrally similar to ribothymidine but differed from the dinucleoside phosphates in that it was not affected by treatment with whole snake venom. Snake venom enzymes induce hydrolysis of each of the known dinucleoside phosphates to give equimolar amounts of an $O^{2'}$-methylnucleoside (Nm), a normal nucleoside (N), and inorganic phosphate. Resistance to the hydrolytic action of whole venom suggested that T? was not a dinucleoside phosphate, even though it had a single negative charge at pH 7, a characteristic of dinucleoside phosphates.

We have found, in fact, that T? is *not* a dinucleoside phosphate, but instead a carboxymethyl-containing nucleoside (see Note Added in Proof, Gray and Lane,

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‡ Present address: University of Toronto, Department of Biochemistry, Toronto, Ontario, Canada; address to which communications in connection with this paper should be sent.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: m_2^6A, N^6,N^6-dimethyladenosine; PME, *E. coli* alkaline phosphatase; UPRP, β-ureidopropionic acid *N*-ribosylphosphate.

[2] The conditions which we use for alkali hydrolysis (1 M NaOH, 90 hr, room temperature) are sufficient to ensure complete hydrolysis of most of those normal oligonucleotides (*e.g.*, ApAp) which are only slowly hydrolyzed by alkali (Lane and Butler, 1959). However, it is relevant to point out that even after such extended hydrolysis of *E. coli* rRNA, the dinucleotide $m_2^6Apm_2^6Ap$ can still be detected in trace quantity (Nichols and Lane, 1966). Two minor side reactions which occur under these conditions of hydrolysis are (1) *N*-glycosyl cleavage, which gives rise to trace amounts of free bases ($k_{\text{first order}}$ = *ca.* 10^{-5} hr^{-1}), and (2) deamination of cytosine residues to uracil residues ($k_{\text{first order}}$ = *ca.* 10^{-3} hr^{-1}).

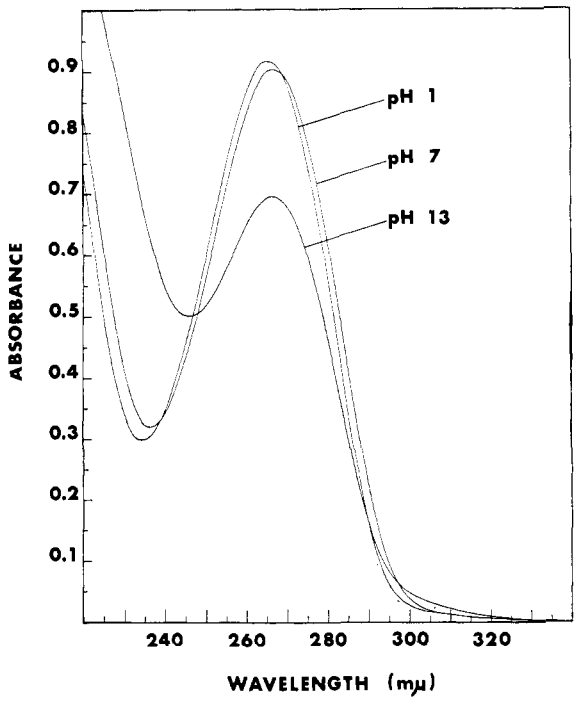

FIGURE 1: Ultraviolet absorption spectra of 5-carboxymethyluridine ("T?") isolated from brewers' yeast tRNA. λ_{max} (mμ) 265 (pH 1), 266.5 (pH 7), and 266.5 (pH 13); λ_{min} (mμ) 234 (pH 1), 236 (pH 7), and 245.5 (pH 13).

1967), derived from 5-carboxymethyluridine 2′(3′)-phosphate during phosphomonoesterase treatment of the alkali-stable dinucleotides from wheat embryo tRNA and yeast tRNA. The carboxyl group imparts an additional negative charge to the nucleoside monophosphate at pH 7.8, and as a result, 5-carboxymethyluridylate, with its three negative charges, is eluted together with the alkali-stable dinucleotides during ion-exchange chromatography on DEAE-cellulose at pH 7.8.

The present report describes the isolation of 5-carboxymethyluridylate from alkali hydrolysates of wheat embryo tRNA and yeast tRNA, and presents evidence in support of the structural assignment.

Materials

Brewers' yeast tRNA was purchased from Boehringer Mannheim Corp. Wheat embryo tRNA was prepared according to the method of Glitz and Dekker (1963), except that after the methyl Cellosolve extraction step, the RNA was recovered by precipitation as the cetyltrimethylammonium salt (Ralph and Bellamy, 1964). *E. coli* tRNA was a product of General Biochemicals.

Dihydrouridine 2′(3′)-phosphate was purchased from Calbiochem. 6-Carboxymethyluracil(uracil-4-(or-6)acetic acid) was obtained from General Biochemicals and Mann Research Laboratories, Inc. All commercial samples of this compound contained 5–10% of an ultraviolet-absorbing contaminant identified as 6-methyluracil. Recrystallization of the commercial material from hot water gave 6-carboxymethyluracil which still contained some 6-methyluracil, since 6-carboxymethyluracil is susceptible to decarboxylation at elevated temperatures. However, 6-methyluracil was removed from the commercial samples of 6-carboxymethyluracil by chromatography of the commercial material on Whatman No. 1 paper (developing solvent = 95% ethyl alcohol–water, 4:1, v/v), or, on a larger scale, by adsorption of the ionized 6-carboxymethyluracil to DEAE-cellulose and elution with 1 M formic acid (pH 1.8).

Thiourea, diethyl succinate, and ethyl formate were purchased from Aldrich Chemical Co., Inc. Pyridine (Spectroquality reagent) was obtained from Matheson Coleman and Bell.

Methods

Melting points were determined on a Hoover capillary melting point apparatus (Arthur H. Thomas Co.), and are uncorrected. Microanalyses were performed by Galbraith Laboratories, Inc. Nuclear magnetic resonance spectra were measured with a Varian Associates Model A-60 spectrometer. All nuclear magnetic resonance spectra reported are proton spectra and chemical shifts are relative to tetramethylsilane as external standard at τ 10. Infrared spectra were determined on a Perkin-Elmer Model 421 spectrophotometer; only the more prominent absorptions are recorded in this report. Ultraviolet spectra were recorded on a Cary Model 15 spectrophotometer. High-resolution mass spectra were determined on an AEI MS-9 mass spectrometer at an ionizing voltage of 70 eV; values in parentheses following mass numbers refer to relative intensities of peaks, and only those peaks with an intensity of at least 10% of the most intense peak are reported. Analyses for β-alanine were carried out on a Beckman Model 120C amino acid analyzer.

Electrophoretic studies were carried out with a Durrum-type paper electrophoresis cell (Model R, Series D; Beckman) at 500 V for 1–2 hr. The systems used for paper chromatography have previously been described in detail (Gray and Lane, 1967). The developing solvents were 95% ethyl alcohol–water (4:1, v/v) (system 1) and saturated ammonium sulfate–2-propanol (40:1, v/v) (system 2); they were used in conjunction with Whatman No. 1 chromatography paper which had been impregnated with ammonium sulfate (Lane, 1963).

Results

General Approach to the Characterization of 5-Carboxymethyluridylate, 5-Carboxymethyluridine, and 5-Carboxymethyluracil Derived from tRNA. The unknown compound (T?) mentioned in the introduction to this paper was very similar to ribothymidine in its ultraviolet spectral properties (Figure 1), and was isolated from a fraction that was initially believed to be composed exclusively of dinucleoside phosphates. Since, as with dinucleoside phosphates, T? had a single negative charge at pH 7, it at first appeared that it might be an homologous dinucleoside phosphate containing two residues of ribothymidine, or perhaps a dinucleoside phosphate composed of ribothymidine and a residue

having negligible ultraviolet absorbance in the 260-mμ region, such as dihydrouridine (Madison and Holley, 1965) or the alkali-conversion product of dihydrouridine, β-ureidopropionic acid *N*-riboside (Sanger *et al.*, 1965). However, snake venom enzymes, which induce hydrolysis of 3′–5′, 2′–5′, and 5′–5′-phosphodiester bonds and P^1,P^2-nucleoside 5′-pyrophosphate bonds, had no effect on T?. It was then conjectured that T? might be a nucleoside bearing a negative charge at pH 7. If T? were a nucleoside, it would of course be unaffected by nonspecific phosphomonoesterase, phosphodiesterase, and pyrophosphatase activities; furthermore, its negative charge at pH 7 could not be due to a primary phosphate dissociation, a view that was substantiated by the finding that the compound was uncharged at pH 1.8. From this result, it seemed that the acquisition of a negative charge between pH 2 and 7 might be due to the ionization of a carboxyl group. Since the nucleoside was similar to ribothymidine in its ultraviolet spectral properties, it was concluded that any carboxyl substituent attached to the *base* would have to be linked to the ring through one or more methylene groups, as direct attachment of a carboxyl group to *any* position of uracil would be inconsistent with the observed similarity between the spectral properties of T? and those of ribothymidine (see, for example, Imai and Honjo (1965) for the ultraviolet absorption spectrum of 5-carboxyuridine). The similarity between the ultraviolet spectra of T? and ribothymidine also suggested that any such substituent would most likely be bonded to the ring at the C-5 position. Alternatively, attachment of a carboxyl group to the *sugar* of the nucleoside would not markedly affect ultraviolet spectral properties. However, attachment of the negatively charged group to the sugar was eliminated as a possibility when a base, possessing the negatively charged group characteristic of T?, was isolated from this compound. This base had ultraviolet spectral properties similar to (but not identical with) those of 5-methyluracil (thymine), and distinctly different from those of 6-methyluracil.

The simplest possibility seemed to be that the *base* in T? had a carboxyl group attached through a methylene substituent at C-5 of uracil. Consequently, 5-carboxymethyluracil was synthesized and compared with the base derived from T?. It was found that this base was electrophoretically, chromatographically, and spectrally identical with synthetic 5-carboxymethyluracil, and was easily distinguishable from commercially available 6-carboxymethyluracil.

The presumed 5-carboxymethyl derivatives of uridylate, uridine, and uracil were prepared from tRNA, and these compounds were then systematically compared, by paper electrophoretic and chromatographic techniques, with 5-methyl (and other) derivatives of uridylate, uridine, and uracil. These studies provide ample evidence of the increased polarity and ionic character expected for the carboxymethyl derivatives of uracil. All of the studies to be reported are consistent with the view that T? is 5-carboxymethyluridine, and that in earlier investigations, it was derived from 5-carboxymethyluridine 2′(3′)-monophosphate during phosphomonoesterase treatment of the alkali-stable dinucleotides from alkali hydrolysates of wheat embryo tRNA and yeast tRNA.

Phosphorus analysis of 5-carboxymethyluridine 2′(3′)-monophosphate and identification of the *sugar* in 5-carboxymethyluridine presented special problems. These problems, together with a description of the obstacles encountered in attempting to isolate 5-carboxymethyluridine 5′-phosphate from a venom phosphodiesterase hydrolysate of tRNA, will be described in the final sections of this report.

The interrelation of the various carboxymethyl derivatives from tRNA is outlined in Figure 2, along with a schematic representation of the synthesis of 5-carboxymethyluracil.

Isolation of 5-Carboxymethyluridine 2′(3′)-Phosphate from Alkali Hydrolysates of tRNA. Before alkali hydrolysis, all tRNA preparations were chromatographed on DEAE-cellulose in order to remove any low molecular weight nucleotides and/or contaminant rRNA (Glitz and Dekker, 1963; Bell *et al.*, 1964). Chemical and physicochemical characterizations of the purified preparations of wheat embryo tRNA and brewers' yeast tRNA have been reported (Hudson *et al.*, 1965; Kay and Oikawa, 1966; Gray and Lane, 1967). The tRNA preparations from wheat embryo and yeast had similar amino acid accepting capacities when assayed with their homologous amino acid activating enzymes (M. W. Gray, unpublished observations). Procedures have been described (Gray and Lane, 1967) for (i) exhaustive alkali hydrolysis of RNA, (ii) isolation of dinucleotides from alkali hydrolysates by chromatography on DEAE-cellulose at pH 7.8, (iii) fractionation of the bulk dinucleotides by chromatography on DEAE-cellulose at pH 1.8, and (iv) recovery of salt-free compounds from column eluates.

Figure 3 shows the elution profiles obtained when the bulk dinucleotides from yeast tRNA (A), wheat embryo tRNA (B), and *E. coli* tRNA (C) were chromatographed on DEAE-formate at pH 1.8. When 5-carboxymethyluridylate was present, it appeared in subfraction 3b, eluting in the same position as the dinucleotides AmpUp and UmpAp, which have a net charge of −1 at pH 1.8. This chromatographic behavior is expected for 5-carboxymethyluridylate, which has a net charge of −1 at pH 1.8 because of the suppression of the secondary phosphate and carboxyl ionizations at this low pH value. The prominence of subfraction 3b in the elution profiles for dinucleotides from yeast tRNA (Figure 3A) reflects (1) the presence of the dinucleotide AmpUp which is found in alkali hydrolysates of yeast tRNA but is not found in alkali hydrolysates of wheat embryo tRNA, and (2) a higher content of 5-carboxymethyluridylate in alkali hydrolysates of yeast tRNA, relative to wheat embryo tRNA.

Paper chromatography was used for the final separation of 5-carboxymethyluridylate from the alkali-stable dinucleotides in subfraction 3. In system 2, 5-carboxymethyluridylate is the fastest moving of the components of subfraction 3, and is well resolved from the fastest moving dinucleotide, CmpUp. The nucleotide was eluted from chromatograms with water, and the resulting aqueous eluate was freed of ammonium sulfate

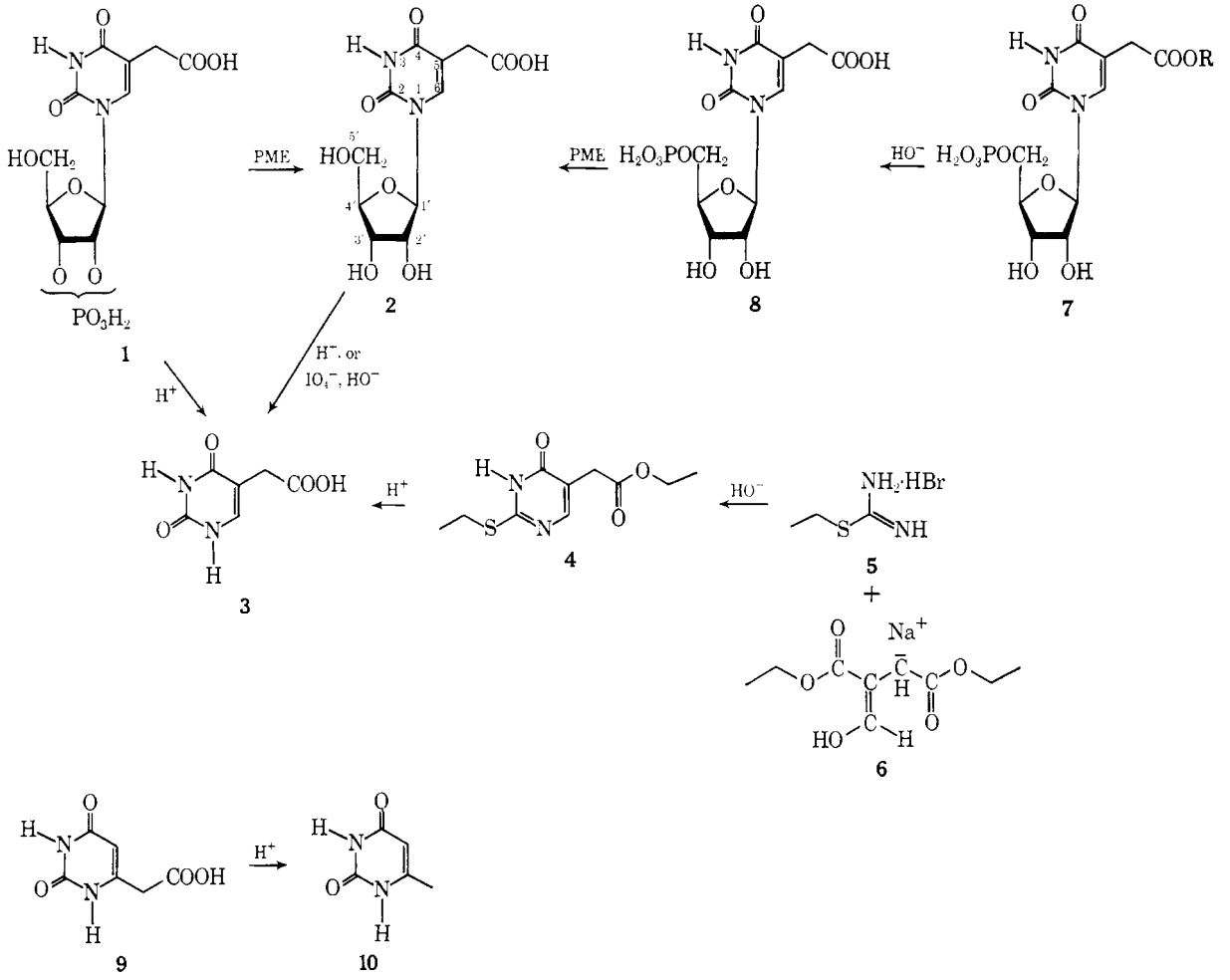

FIGURE 2: Diagram illustrating the synthesis of 5-carboxymethyluracil, and the structures and interrelations of the various carboxymethyluracil derivatives. The compounds are: 5-carboxymethyluridine 2′(3′)-phosphate (cm^{5}Up) (**1**), 5-carboxymethyluridine (cm^{5}U) (**2**), 5-carboxymethyluracil (cm^{5}u) (**3**), ethyl 2-ethylthio-4-oxypyrimidine-5-acetate (**4**), *S*-ethylthiourea hydrobromide (**5**), diethyl formylsuccinate, sodium salt (**6**), unspecified carboxylate ester of 5-carboxymethyluridine 5′-phosphate (**7**), 5-carboxymethyluridine 5′-phosphate (pcm^{5}U) (**8**), 6-carboxymethyluracil (cm^{6}u) (**9**), and 6-methyluracil (m^{6}u) (**10**).

derived from the developing solvent) by passage through DEAE-carbonate according to the method of Rushizky and Sober (1962). After exhaustive washing of the column with 0.01 M ammonium carbonate, in order to remove sulfate ions, the ammonium carbonate eluent was in turn removed from the column by a water wash. The 5-carboxymethyluridylate was then eluted from the column with 1 M pyridinium formate (pH 4.5), which is more easily removed *in vacuo* than are ammonium carbonate eluents. Essentially quantitative recovery of salt-free 5-carboxymethyluridylate could be achieved by the procedure just described, and the product was free of other ultraviolet-absorbing materials, as judged by the results of paper chromatography and electrophoresis in several different systems.

When desalting with charcoal was attempted, it was found that 5-carboxymethyluridylate could not be quantitatively desorbed from charcoal after the removal of salt. We have observed (Diemer, 1965) that nucleosides and nucleoside monophosphates can be adsorbed to charcoal and then desorbed, with 80–90% recovery, regardless of the ionic strength of the weakly acidic solutions (pH 4–5) in which these compounds are applied to charcoal columns. However, in the case of nucleoside 2′(3′),5;-bisphosphates, nearly 50% of the nucleoside diphosphate fails to adsorb to the charcoal from salt-free solution, while at ionic strengths greater than or equal to 0.05 M, about 30–40% of the nucleoside diphosphate is irreversibly bound to the charcoal. Presumably because of its additional acidic carboxyl function, 5-carboxymethyluridylate behaves in the same way as nucleoside 2′(3′),5-bisphosphates when passed through charcoal disks.

Derivation of 5-Carboxymethyluridine and 5-Carboxymethyluracil from the 5-Carboxymethyluridylate of tRNA. The *nucleoside* of 5-carboxymethyluridylate was prepared by treatment of the parent nucleotide **1** with *E. coli* alkaline phosphatase under the conditions used in previous work with dinucleotides (Gray and Lane, 1967). The nucleoside **2** obtained in this manner was indistinguishable from the compound T? mentioned earlier in this paper. The ultraviolet absorption spectra of 5-carboxymethyluridine isolated from brewers' yeast tRNA are shown in Figure 1.

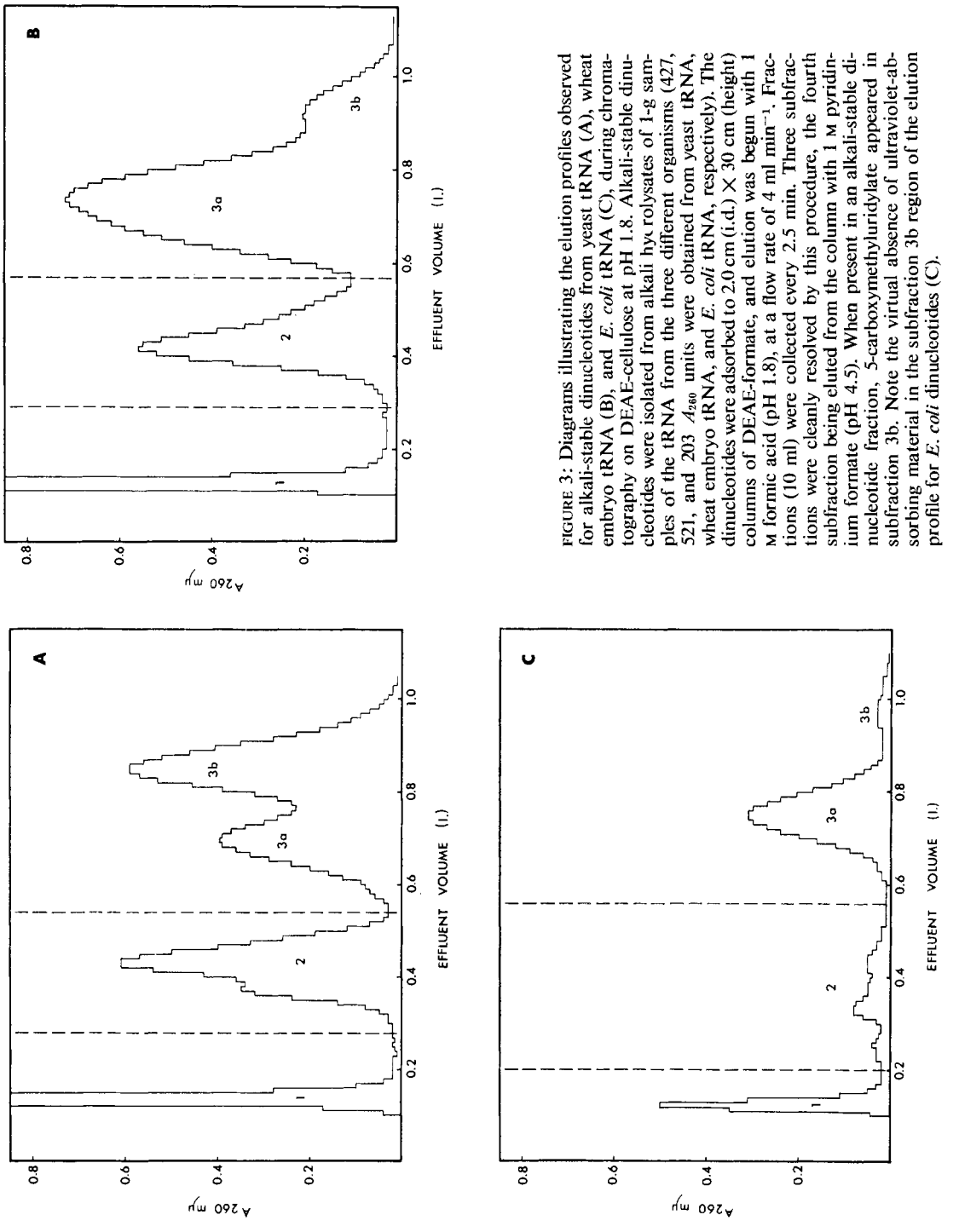

FIGURE 3: Diagrams illustrating the elution profiles observed for alkali-stable dinucleotides from yeast tRNA (A), wheat embryo tRNA (B), and *E. coli* tRNA (C), during chromatography on DEAE-cellulose at pH 1.8. Alkali-stable dinucleotides were isolated from alkali hydrolysates of 1-g samples of the tRNA from the three different organisms (427, 521, and 203 A_{260} units were obtained from yeast tRNA, wheat embryo tRNA, and *E. coli* tRNA, respectively). The dinucleotides were adsorbed to 2.0 cm (i.d.) × 30 cm (height) columns of DEAE-formate, and elution was begun with 1 M formic acid (pH 1.8), at a flow rate of 4 ml min^{-1}. Fractions (10 ml) were collected every 2.5 min. Three subfractions were cleanly resolved by this procedure, the fourth subfraction being eluted from the column with 1 M pyridinium formate (pH 4.5). When present in an alkali-stable dinucleotide fraction, 5-carboxymethyluridylate appeared in subfraction 3b. Note the virtual absence of ultraviolet-absorbing material in the subfraction 3b region of the elution profile for *E. coli* dinucleotides (C).

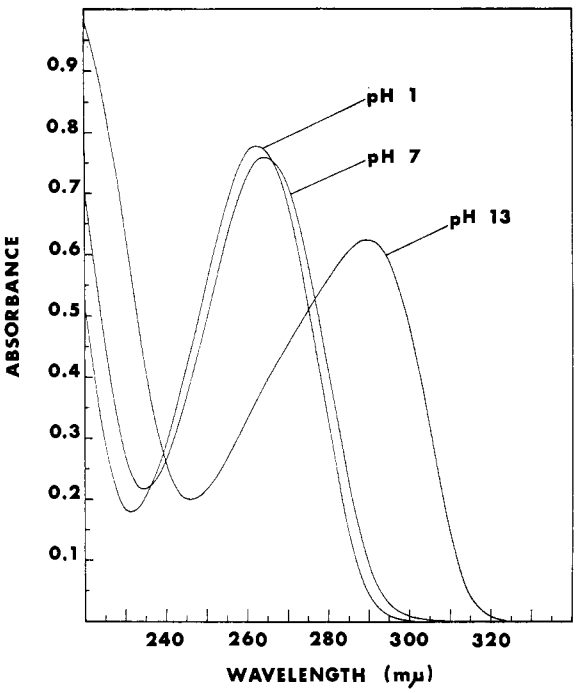

FIGURE 4: Ultraviolet absorption spectra of "natural" 5-carboxymethyluracil, obtained by acid hydrolysis of 5-carboxymethyluridylate which had been isolated from wheat embryo tRNA.

The *base* (**3**) of 5-carboxymethyluridine was prepared by treatment of the nucleoside with periodate and incubation of the resulting dialdehyde at alkaline pH, as described by Neu and Heppel (1964). Alternatively, and more conveniently, the base was prepared by hydrolysis of the parent nucleotide or nucleoside in 6 M HCl for 6 hr at 100°, in a sealed, evacuated tube (Furukawa *et al.*, 1965). The ultraviolet absorption spectra of 5-carboxymethyluracil isolated from wheat embryo tRNA are shown in Figure 4.

Synthesis and Characterization of 5-Carboxymethyluracil. 5-Carboxymethyluracil was prepared as described by Johnson and Speh (1907). The synthesis is outlined in Figure 2 and initially involves the condensation of *S*-ethylthiourea hydrobromide (**5**) with the sodium salt of diethyl formylsuccinate (**6**) to yield ethyl 2-ethylthio-4-oxypyrimidine-5-acetate (**4**). This pyrimidine is then quantitatively converted into 5-carboxymethyluracil (**3**) by refluxing with hydrochloric acid.

After decolorizing with charcoal, ethyl 2-ethylthio-4-oxypyrimidine-5-acetate crystallized from ethanol in the form of slender, colorless needles: mp 146.0–146.5°, lit. (Johnson and Speh, 1907) mp 146–147°. The product migrated as a single ultraviolet-absorbing spot (R_F *ca.* 0.9) during paper chromatography in system 1.

Anal. Calcd for $C_{10}H_{14}N_2O_3S$: C, 49.57; H, 5.82; N, 11.56; S, 13.23; mol wt, 242.0725. Found: C, 49.35; H, 5.99; N, 11.58; S, 13.40; mol wt, 242.0725 (mass spectrometry).

The nuclear magnetic resonance spectrum (τ) (acetone-d_6) showed 2.60 (1 H, singlet, H-6), 6.37 (2 H, quartet, OCH_2CH_3, J = 7 cps), 7.08 (2 H, singlet, $=CCH_2C(=O)$), 7.29 (2 H, quartet, SCH_2CH_3, J = 7 cps), 9.17 and 9.30 (6 H, OCH_2CH_3 and SCH_2CH_3, two overlapping triplets, J = 7 cps). The infrared spectrum showed λ_{max}^{Nujol} (μ) 5.81 and 5.83 (carbonyl stretching; $CH_2C(=O)OCH_2$), 5.99–6.05 (carbonyl stretching; C(=O)NH), 6.44, 7.32, 7.45, 7.86, 8.28, 8.40, 8.62, 9.73, and 10.25. The mass spectrum (direct probe, source temperature 175°) showed *m/e* 242 (M^+; 50), 214 (16), 197 (13), 170 (12), 169 (100), 168 (39), 141 (19), 82 (82), 55 (20), 54 (12), 39 (12), 29 (47), 28 (10), and 27 (21).

5-Carboxymethyluracil was obtained from ethyl 2-ethylthio-4-oxypyrimidine-5-acetate by refluxing a solution of 5 g of the thiopyrimidine in 50–100 ml of concentrated hydrochloric acid. After decolorizing with charcoal, 5-carboxymethyluracil crystallized from boiling water in the form of white, granular crystals, mp 311.0–311.5°, with decomposition (lit. (Johnson and Speh, 1907) mp 315–320°, with decomposition). Yields were quantitative (>95%). The final product was homogeneous in several different chromatography and electrophoresis systems.

Anal. Calcd for $C_6H_6N_2O_4$: C, 42.36; H, 3.56; N, 16.47; mol wt, 170.0328. Found: C, 41.89; H, 3.57; N, 16.32; mol wt, 170.0328 (mass spectrometry).

The nuclear magnetic resonance spectrum (τ) (D_2O) showed 2.40 (1 H, singlet, H-6) and 6.75 (2 H, singlet, $>CCH_2C(=O)$. The infrared spectrum showed λ_{max}^{Nujol} (μ) 5.78 (carbonyl stretching; $CH_2C(=O)OH$), 5.91 and 6.00 (carbonyl stretching; C(=O)NH), 6.77, 7.03, 7.95, 8.39, and 13.3. The mass spectrum (direct probe, source temperature 200°) showed *m/e* 170 (M^+; 2), 126 ($-CO_2$; 100), 125 (18), 83 (19), 82 (88), 55 (70), 54 (35) 53 (14), 52 (16), 39 (11), 29 (52), 28 (27), 26 (12), and 18 (14). The ultraviolet spectrum (see Figure 5) showed λ_{max} (mμ) 262 (pH 1), 264 (pH 7), and 290 (pH 13); λ_{min} (mμ) 231 (pH 1), 234 (pH 7), and 246 (pH 13); ϵ_{260} 7240 in 0.01 M phosphate buffer (pH 7.45).

Characterization of 6-Carboxymethyluracil and Comparison with Synthetic 5-Carboxymethyluracil. Commercially available 6-carboxymethyluracil was freed from traces of 6-methyluracil as described in Materials.

Anal. Calcd for $C_6H_6N_2O_4$: mol wt, 170.0328. Found: mol wt, 170.0328 (mass spectrometry).

The nuclear magnetic resonance spectrum (D_2O) showed τ 4.13 (1 H, singlet, H-5). The resonance signal expected for the methylene protons of the carboxymethyl side chain was virtually absent in the nmr spectrum of 6-carboxymethyluracil in D_2O; only a very small residual peak was evident at τ 6.46 (the analogous resonance signal occurs at τ 6.75 in the case of 5-carboxymethyluracil). Since the DOH signal of the 6 isomer integrated for two protons more than the DOH signal of the 5 isomer, it would appear that the methylene protons of the carboxymethyl side chain of 6-carboxymethyluracil are exchangeable with deuterium.

The infrared spectrum showed λ_{max}^{Nujol} (μ) 5.82 (carbonyl stretching; $CH_2C(=O)OH$), 5.94 and 6.04–6.09 (carbonyl stretching; C(=O)NH), 6.64, 7.07, 8.18, and 12.2. The mass spectrum (direct probe, source temperature 200°) showed *m/e* 170 (M^+; 1), 126 (loss of CO_2; 95), 83 (44), 68 (44), 44 (100), 42 (87), 41 (17), 40 (12), 39 (10), 28 (12), 27 (11), and 18 (42). The ultraviolet spectrum (see Figure 6) showed λ_{max} (mμ) 261

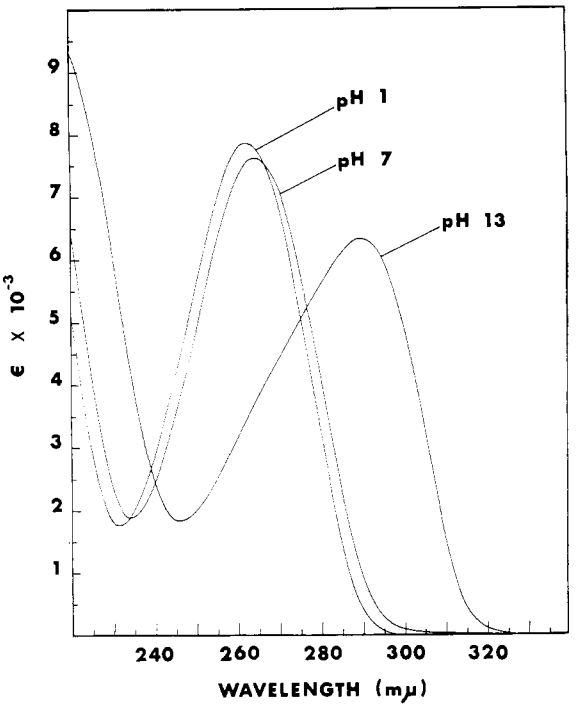

FIGURE 5: Ultraviolet absorption spectra of synthetic 5-carboxymethyluracil.

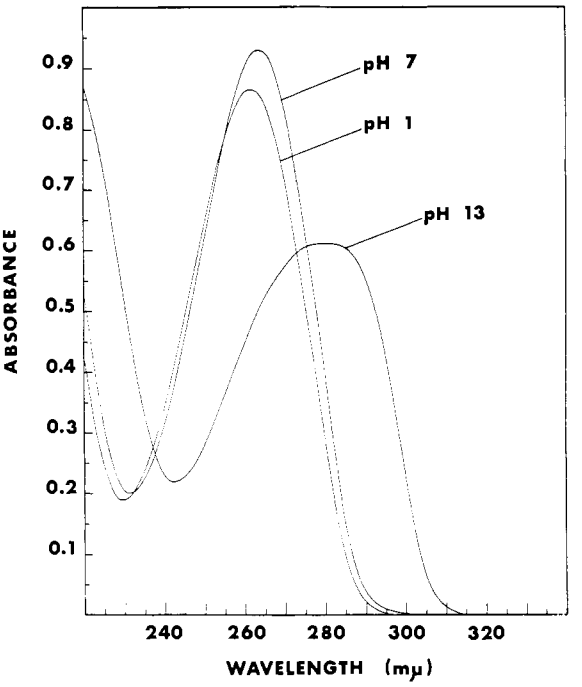

FIGURE 6: Ultraviolet absorption spectra of 6-carboxymethyluracil.

(pH 1), 263 (pH 7), and 276.5–283 (pH 13); λ_{min} (mμ) 229 (pH 1), 231 (pH 7), and 242 (pH 13).

5-Carboxymethyluracil (**3**) and 6-carboxymethyluracil (**9**) can be readily distinguished from one another on the basis of their nuclear magnetic resonance, infrared, and ultraviolet spectra. Of particular note are the nuclear magnetic resonance spectra, where the chemical shifts for the vinylic protons at C-5 and C-6 serve to establish the position of attachment of the carboxymethyl side chains to the pyrimidine ring. The resonance signals for the C-5 and C-6 protons of uracil, in D_2O, occur at τ 4.29 and 2.40, respectively (Bhacca *et al.*, 1963), and since the nuclear magnetic resonance spectrum of 5-carboxymethyluracil shows only the signal at τ 2.40, the C-5 proton must be absent. In the case of 6-carboxymethyluracil, the lone resonance signal at τ 4.13 establishes the presence of a C-5 proton and the absence of a C-6 proton.

The infrared spectra of the two isomers are somewhat similar in the 6-μ region (carbonyl stretching absorptions), but the absorption patterns in the fingerprint region (7–11 μ) are quite distinct. Although obscured somewhat by the C–H stretching absorptions of Nujol, the broad, medium-intensity absorption indicative of a carboxylic acid functional group is still discernible in the 3.0–3.5-μ region in the infrared spectra of both 5-carboxymethyluracil and 6-carboxymethyluracil.

The ultraviolet spectra of 5-carboxymethyluracil (Figure 5) and 6-carboxymethyluracil (Figure 6) also show characteristic differences, most notably at pH 13. The A_{290}/A_{260} ratio at pH 13 is 2.0 in the case of 5-carboxymethyluracil, but only 1.2 in the case of 6-carboxymethyl uracil. Other uracil derivatives, such as 5-methyluracil and 6-methyluracil, as well as uracil itself, have A_{290}/A_{260} ratios of less than 1.5 at pH 13.

The high-resolution mass spectra of both 5-carboxymethyluracil and 6-carboxymethyluracil show an intense peak at *m/e* 126, corresponding to loss of carbon dioxide from the carboxymethyl side chains of the parent compounds. The exact masses of the parent peaks at *m/e* 170 were measured, and, as expected, were found to be identical.

Comparison of 5- and 6-Carboxymethyluracil with the Base Derived from tRNA. The base isolated by acid hydrolysis of the presumed 5-carboxymethyluridylate (from either yeast or wheat embryo tRNA) had ultraviolet spectra (Figure 4) virtually identical with those of synthetic 5-carboxymethyluracil (Figure 5) at pH values of 1, 7, and 13 (Figure 5), and quite different from those of 6-carboxymethyluracil (Figure 6) at these same pH values. Either acid hydrolysis or periodate treatment of 5-carboxymethyluridine from tRNA also gave rise to a base having an ultraviolet spectrum identical with that of 5-carboxymethyluracil.

The base derived from tRNA was also identical with 5-carboxymethyluracil in its electrophoretic and chromatographic behavior (Tables I and II). In several systems, the "natural" base and synthetic 5-carboxymethyluracil had a mobility markedly different from that of 6-carboxymethyluracil. During electrophoresis at pH 3.5, for example, 6-carboxymethyluracil migrated twice as fast as 5-carboxymethyluracil and the natural base, indicating that the 6 isomer is a substantially stronger acid than the 5 isomer.

The natural base also paralleled 5-carboxymethyluracil in its behavior toward treatment with acid. Synthetic 5-carboxymethyluracil was stable under the con-

TABLE I: Relative Electrophoretic Mobilities of Various Uracil Derivatives.[a]

Compound	Buffer pH 1.8	3.5	5.0	9.2 (formate)	9.2 (borate)
Up	+0.51	+0.52	+0.77	+1.30	+1.21
cm^{5}Up (yeast tRNA)	+0.45	+0.63	+1.30	+1.65	+1.58
cm^{5}Up (wheat embryo tRNA)	+0.49	+0.67	+1.36	+1.70	+1.63
m^{5}Up	+0.54	+0.51	+0.59	+1.19	+1.06
Ψp	+0.45	+0.53	+0.64	+1.35	+1.21
pU	+0.55	+0.59	+0.67	+1.35	+1.48
U	−0.07	−0.05	−0.19	−0.04	+0.52
cm^{5}U (yeast tRNA)	−0.08	+0.25	+0.51	+0.53	+1.02
cm^{5}U (wheat embryo tRNA)	−0.05	+0.22	+0.52	+0.52	+1.04
m^{5}U	−0.07	−0.05	−0.15	−0.11	+0.51
Ψ	−0.05	−0.05	−0.16	0.00	+0.55
u	−0.05	−0.04	−0.09	−0.04	+0.32
cm^{5}u (yeast tRNA)	−0.03	+0.31	+0.86	+1.13	+0.92
cm^{5}u (wheat embryo tRNA)	−0.04	+0.31	+0.87	+1.14	+0.98
cm^{5}u (synthetic)	−0.05	+0.31	+0.80	+1.10	+0.92
cm^{6}u	−0.03	+0.75	+0.96	+1.19	+1.13
m^{5}u	−0.04	+0.02	−0.15	−0.11	0.00
m^{6}u	−0.04	−0.10	−0.15	−0.07	+0.08

[a] The picrate marker was assigned a mobility of +1.00 at each pH value. Positive values indicate that migration was toward the anode, and negative values indicate migration toward the cathode. The buffers used were: (A) 1 M formic acid (pH 1.8), (B) 0.025 M ammonium formate (pH 3.5), (C) 0.025 M ammonium formate (pH 5.0), (D) 0.025 M ammonium formate (pH 9.2), and (E) 0.025 M sodium (tetra)borate (pH 9.2). Compounds (*ca.* 0.05 μmole, in 25 μl) were spotted on 3.0 × 30.5 cm strips of Whatman No. 1 filter paper impregnated with the appropriate buffer. The symbols "U" and "Ψ" are abbreviations for the nucleosides uridine and pseudouridine, respectively, and the symbol "u" is an abbreviation for the base, uracil. The prefix "cm" is used to designate a carboxymethyl group, whereas "m" designates a methyl group. The superscripts following these prefixes refer to the position of the ring at which substitution has occurred, *e.g.*, "cm^{5}U" is 5-carboxymethyluridine.

ditions of acid hydrolysis used for the production of the natural base from 5-carboxymethyluridylate or 5-carboxymethyluridine (6 N HCl, 100°, 6 hr), since more than 95% of the compound could be recovered unchanged after such treatment. No 5-methyluracil was detectable in acid hydrolysates of either the natural base or synthetic 5-carboxymethyluracil, indicating that decarboxylation does not occur to any significant extent under these conditions. The acid stability of 5-carboxymethyluracil has previously been noted (Johnson and Speh, 1907). In marked contrast, when 6-carboxymethyluracil was treated with acid under the above conditions, more than 95% of this compound was decarboxylated to form 6-methyluracil (**10**), as indicated in Figure 2. These results are consistent with the view that carboxymethyl substitution occurs at C-5 and not C-6 of uracil in the naturally occurring compound.

Paper Electrophoretic Characterization of 5-Carboxymethyluridylate, 5-Carboxymethyluridine, and 5-Carboxymethyluracil Derived from tRNA. The electrophoretic mobilities listed in Table I show that above pH 3, 5-carboxymethyluridylate has a greater negative charge than 5-methyluridylate or uridylate. Removal of monoester phosphate groups abolishes the mobility imparted to each nucleotide by the ionized primary phosphate at pH 1.8 and by the ionized primary and secondary phosphates above pH 5. However, 5-carboxymethyluridine and 5-carboxymethyluracil continue to display a negative charge above pH 3, whereas uridine and 5-methyluridine, as well as their bases, are uncharged at pH values less than 9. As expected for 5-carboxymethyluridine and its base, acquisition of a negative charge occurs over the range of pH 3–5 ($pK_a^{25°}$ = 4.3 for phenylacetic acid). Thus, column chromatography with urea-containing eluents had indicated that 5-carboxymethyluridylate had a net charge of −3 at pH 7.8, and these electrophoretic studies are consistent with the view that the charges at pH 7.8 arise from one carboxyl, one primary phosphate, and one secondary phosphate ionization.

Electrophoresis of 5-carboxymethyluridine in the presence and absence of borate at pH 9.2 shows that the sugar moiety contains a *cis*-diol grouping, since the mobility of the nucleoside is greatly enhanced in the presence of borate. On the other hand, the mobility of the parent nucleotide at pH 9.2 is not affected by the presence of borate. Since this nucleotide was isolated from an alkali hydrolysate of tRNA, these results are

consistent with the expectation that in the parent nucleotide, a phosphomonoester group is attached at the 2′ or 3′ position of a ribose residue.

Paper Chromatographic Characterization of 5-Carboxymethyluridylate, 5-Carboxymethyluridine, and 5-Carboxymethyluracil Derived from tRNA. The chromatographic mobilities listed in Table II show that the 5-carboxymethyl derivatives of uridylate, uridine, and uracil migrate more slowly than the corresponding 5-methyl derivatives in a relatively nonpolar solvent system (system 1), but migrate more rapidly than the corresponding 5-methyl derivatives in a relatively polar solvent system (system 2). These observations are consistent with the greater polarity expected for 5-carboxymethyl derivatives, relative to the corresponding nonpolar 5-methyl derivatives of uracil.

The 5-carboxymethyluridylate from alkali hydrolysates of tRNA partially resolves into two spots when chromatographed in system 2, and this resolution is analogous with the resolution of the 2′- and 3′-nucleotides of uridine and 5-methyluridine in this same system. This is consistent with the view that 5-carboxymethyluridylate from alkali hydrolysates of tRNA is an isomeric mixture of nucleotides in which phosphate is esterified at either the 2′ or 3′ position of a ribose residue.

Phosphorus Analysis of 5-Carboxymethyluridylate Derived from Alkali Hydrolysates of tRNA. Since it was found that the molar extinction coefficients of 5-carboxymethyluracil and thymine were similar ($\epsilon_{260}^{pH\,1}$ 7750 and 7400, respectively), and since the ultraviolet spectrum of 5-carboxymethyluridine resembled that of ribothymidine, it seemed probable that 5-carboxymethyluridine, and its nucleotide, would have molar extinction coefficients similar to that of ribothymidine (ϵ_{260} *ca.* 8000). Assuming this to be the case, it was surprising to find that 5-carboxymethyluridylate isolated from wheat embryo tRNA contained 2.1 moles of phosphorus/mole of nucleotide, and that after dephosphorylation, the resulting 5-carboxymethyluridine consumed *ca.* 2 moles of periodate/mole of nucleoside. Before the structure of 5-carboxymethyluridylate was established, these data suggested that the unknown nucleotide might be a diphosphorylated diribosyl derivative of uracil (Lis and Lis, 1962), as was previously discussed (Hudson *et al.*, 1965). However, it was found that 5-carboxymethyluridylate from yeast tRNA, which was isolated by the procedure described earlier in this paper and which was electrophoretically and chromatographically identical with the compound from wheat embryo tRNA, contained 3.8 moles of phosphorus/mole of nucleotide. From this result, it seemed likely that there was spurious contamination of our samples of 5-carboxymethyluridylate with one or more phosphorus-containing impurities which did not have appreciable ultraviolet absorbance in the 260-mμ region.

After Madison and Holley (1965) reported the presence of dihydrouridylate in yeast tRNA, it became possible to speculate that at least one of the nonultraviolet-absorbing, phosphorus-containing impurities in 5-carboxymethyluridylate specimens might be UPRP, the alkali-conversion product of dihydrouridylate. The ureido derivative has been shown to be formed from dihydrouridylate in alkaline environments (Cohn and Doherty, 1956). Because UPRP has a single carboxyl group ($pK_a = 4.1$; Sanger *et al.*, 1965) and a single phosphomonoester group, it seemed not unlikely that it might migrate with 5-carboxymethyluridylate during the various ion-exchange and paper chromatographic procedures used in the isolation of 5-carboxymethyluridylate.

After further investigation, it was found that UPRP accounted for *ca.* 70% of the phosphorus in samples of 5-carboxymethyluridylate isolated from brewers' yeast tRNA. Passage through a charcoal disk (Lane and Tamaoki, 1967) removed most of the UPRP from 5-carboxymethyluridylate.[8] The phosphorus-containing material which did not adsorb to charcoal (mainly UPRP) was reactive in the orcinol test (Mejbaum, 1939), in

TABLE II: Relative Chromatographic Mobilities of Various Uracil Derivatives.

Compound	System 1 R_{Up}	System 2 R_F
Up	1.00	0.55, 0.60[a]
cm⁵Up (yeast tRNA)	0.88	0.62, 0.66[a]
cm⁵Up (wheat embryo tRNA)	0.86	0.62, 0.66[a]
m⁵Up	1.12	0.45, 0.53[a]
Ψp	0.60	0.56, 0.63[a]
pU	0.85	0.55
pcm⁵U (wheat embryo tRNA)	0.74	0.62
U	1.25	0.51
cm⁵U (yeast tRNA)	1.18	0.56
cm⁵U (wheat embryo tRNA)	1.18	0.57
m⁵U	1.42	0.41
Ψ	0.86	0.51
u	1.25	0.43
cm⁵u (yeast tRNA)	1.28	0.53
cm⁵u (wheat embryo tRNA)	1.30	0.53
cm⁵u (synthetic)	1.28	0.53
cm⁶u	1.34	0.45
m⁵u	1.50	0.34
m⁶u	1.48	0.37

[a] Resolution into 2′ and 3′ isomers occurred in this system.

[8] For an aqueous solution containing *ca.* 0.1 μmole of 5-carboxymethyluridylate/ml, the best resolution was achieved at a ratio of 10 mg of charcoal/μmole of 5-carboxymethyluridylate. Under these conditions, considerable 5-carboxymethyluridylate did not adsorb to the column, but this loss was acceptable in view of the fact that control experiments indicated that increasing amounts of UPRP were adsorbed to charcoal at higher charcoal to nucleotide ratios.

TABLE III: Proportion of Dihydrouridine and 5-Carboxymethyluridine in the RNA of Various Organisms.

Source and Type of RNA	Proportion of Minor Component (moles/100 moles of constituent nucleosides) Dihydrouridine[a] This Report	Dihydrouridine[a] Magrath and Shaw (1967)	5-Carboxymethyluridine[b]
Yeast tRNA	3.0[c]	3.6	0.34
E. coli tRNA	2.2	2.2	*d*
Wheat embryo tRNA	1.9		0.15
Yeast rRNA		0.02–0.04	*d*
E. coli rRNA		0.02–0.04	*d*
Wheat embryo rRNA	*d*		*d*

[a] Estimated by the procedure of Magrath and Shaw (1967). [b] Estimated as 5-carboxymethyluridylate recovered from alkali hydrolysates. [c] This value refers specifically to *brewers'* yeast tRNA. [d] Not detectable.

agreement with the observation of Cohn and Doherty (1956) that the *N*-ribosyl bond in UPRP is quickly hydrolyzed in strong acid. The unadsorbed material also yielded the expected amount of β-alanine when treated with alkali according to the procedure of Magrath and Shaw (1967), and it cochromatographed and coelectrophoresed with the product obtained by alkali treatment of dihydrouridylate. Both the contaminant material and the alkali-conversion product of dihydrouridine gave the characteristic yellow color with *p*-dimethylaminobenzaldehyde (Fink *et al.*, 1956).

The phosphorus-containing material which adsorbed to charcoal consisted mainly of 5-carboxymethyluridylate, although a continuing but much smaller contamination with UPRP was indicated by the fact that the charcoal-purified 5-carboxymethyluridylate showed a slight reactivity in the orcinol reaction and toward *p*-dimethylaminobenzaldehyde. In addition, the purified nucleotide had a phosphorus/base ratio slightly greater than unity (*ca.* 1.3), while a small amount of β-alanine (*ca.* 0.3 mole/mole of nucleotide) was produced after alkali treatment of the purified nucleotide according to the method of Magrath and Shaw (1967).

Table III gives the proportion of dihydrouridine and 5-carboxymethyluridine in brewers' yeast, *E. coli*, and wheat embryo tRNA. It can be seen that in both yeast tRNA and wheat embryo tRNA, the dihydrouridine content is about tenfold greater than the 5-carboxymethyluridine content. Since control experiments have indicated that UPRP, formed rapidly from dihydrouridylate in alkali, is relatively stable to further breakdown under the conditions which we use for alkali hydrolysis of tRNA, it appears that only a small portion of the UPRP in alkali hydrolysates of yeast and wheat embryo tRNA is isolated along with 5-carboxymethyluridylate by the procedure described earlier.

From the foregoing results, it is clear that the nucleotide described in this report does not contain more than one covalently linked phosphate group per molecule, in accord with its charge properties as revealed by chromatography and electrophoresis, and that the extra phosphate in 5-carboxymethyluridylate samples isolated by our procedure is probably all contributed by a single nonultraviolet-absorbing contaminant, UPRP, the alkali-conversion product of dihydrouridylate.

Characterization of the Sugar in 5-Carboxymethyluridine Isolated from tRNA. The presumed 5-carboxymethyluridylate isolated from tRNA was not affected by treatment with sodium periodate, indicating the absence of a *vic*-diol grouping in the nucleotide. Removal of the phosphomonoester group of the nucleotide, by treatment with PME, led to the appearance of a periodate-susceptible *vic*-diol grouping in the nucleoside product. This behavior is expected when a 2′(3′)-ribonucleotide is converted into the corresponding ribonucleoside. In addition to the acquired sensitivity to periodate oxidation, the nucleoside was now able to complex with borate, a property not shown by the nucleotide from which it was derived.

Since the carboxymethyl nucleoside was derived from RNA, and since its constituent nucleoside was shown to possess a *cis*-diol grouping, it seemed likely that the sugar moiety was ribose. An attempt was made to isolate the sugar from a 5-carboxymethyluridylate sample in which contaminating UPRP had been reduced to a low level (*ca.* 0.3 mole/mole of nucleotide). The purified nucleotide was treated with PME and the resulting nucleoside chromatographed on Whatman No. 1 paper (developing solvent = 95% ethyl alcohol–water, 4:1, v/v). The ultraviolet-absorbing area corresponding to 5-carboxymethyluridine gave a faint positive test with *p*-dimethylaminobenzaldehyde, indicating the presence of a small amount of a ureido derivative, most probably β-ureidopropionic acid *N*-riboside (formed by dephosphorylation of UPRP by PME). After treatment of purified 5-carboxymethyluridine with hydrazine (Littlefield and Dunn, 1958), the sugar liberated was found to be chromatographically identical with D-ribose in system 1, and it is significant that ribose was the only sugar detected when the purified nucleoside was treated with hydrazine. On a semiquantitative basis, the yield of ribose was at least fourfold greater than the amount of

this sugar that could have been expected to be derived from the ureido contaminant, which was treated with hydrazine in a control experiment. Ribose was also the only sugar derived from the ureido compound, and was presumably released by thermal cleavage of the *N*-glycosyl bond at the elevated temperature used for hydrazinolysis (a hydrazine addition product would not be expected to be formed in the case of the ureido compound).

Occurrence of 5-Carboxymethyluridylate in Alkali Hydrolysates of RNA. To date, we have isolated 5-carboxymethyluridylate from the tRNA of bakers' yeast, brewers' yeast, and wheat embryo. As shown in Table III, 5-carboxymethyluridine accounts for 0.34 and 0.15 mole % of the constituent nucleosides in brewers' yeast tRNA and wheat embryo tRNA, respectively, assuming that the molar extinction coefficient of 5-carboxymethyluridine is 8000 at 260 mμ. The data in Tables I and II illustrate that the 5-carboxymethyluridylate isolated from yeast tRNA has the same electrophoretic and chromatographic properties as that isolated from wheat embryo tRNA. 5-Carboxymethyluridylate could not be detected in alkali hydrolysates of *E. coli* tRNA, nor has it been found in alkali hydrolysates of rRNA from wheat embryo (Singh and Lane, 1964b), yeast (Singh and Lane, 1964a; Gray and Lane, 1967), *E. coli* (Nichols and Lane, 1966; Nichols, 1967), or L cells (Lane and Tamaoki, 1967).

Isolation of 5-Carboxymethyluridine 5′-Phosphate from tRNA. To check the possibility that 5-carboxymethyluridine might be present as the free carboxylic acid in tRNA, wheat embryo tRNA was hydrolyzed with purified snake venom phosphodiesterase under conditions that achieve complete hydrolysis of the RNA (Lane *et al.*, 1963; Hudson *et al.*, 1965). The hydrolysis products were fractionated on DEAE-cellulose according to net charge at pH 7.8, and the fraction which eluted after the 5′-nucleotides was examined to determine if it contained any 5-carboxymethyluridine 5′-phosphate. No material that could have been 5-carboxymethyluridine 5′-phosphate was found. However, alkali treatment of the bulk 5′-nucleotides gave a compound having the properties expected of 5-carboxymethyluridine 5′-phosphate. Thus, when the alkali-treated 5′-nucleotides were fractionated on DEAE-cellulose at pH 7.8, the presumed 5-carboxymethyluridine 5′-phosphate was eluted after the other 5′-nucleotides had been completely removed from the column.

The presumed 5′-nucleotide of 5-carboxymethyluridine accounted for *ca.* 0.1 mole % of the alkali-treated 5′-nucleotides, a yield comparable with the amount of 5-carboxymethyluridine 2′(3′)-phosphate found in alkali hydrolysates of wheat embryo tRNA (0.15 mole %). The compound was characterized as 5-carboxymethyluridine 5′-phosphate on the basis of the following observations: (i) the ultraviolet absorption spectrum was similar to that of 5-carboxymethyluridine; (ii) during paper chromatography in system 1, in which 5′-nucleotides migrate more slowly than the corresponding 2′ and 3′ isomers, the compound had an R_F value less than that of 5-carboxymethyluridine 2′(3′)-phosphate (Table II); (iii) during paper chromatography in system 2, the compound migrated as a single ultraviolet-absorbing component, whereas the 5-carboxymethyluridylate isolated from alkali hydrolysates of tRNA partially resolved into its 2′ and 3′ isomers; as with the nucleotide isomers of uridine, the 5′-nucleotide of 5-carboxymethyluridine had an R_F value similar to that of the slower moving (presumably 3′-) isomer from alkali hydrolysates; (iv) the compound migrated slightly behind 2′(3′)-uridylate during paper electrophoresis at pH 1.8 (1 M formic acid), but well ahead of 2′(3′)-uridylate at pH 9.2 (0.025 M ammonium formate or 0.025 M sodium borate); (v) the compound reacted with periodate, indicating the presence of a *vic*-diol grouping, as expected for a 5′-nucleotide; (vi) upon treatment with PME, the compound was converted into a derivative electrophoretically and chromatographically indistinguishable from the 5-carboxymethyluridine produced by PME treatment of 5-carboxymethyluridine 2′(3′)-phosphate.

Discussion

Since our initial report of the isolation of 5-carboxymethyluridine from yeast tRNA (Gray and Lane, 1967), a second carboxymethyl-substituted nucleoside has been found in yeast tRNA by Baczynskyj *et al.* (1968). These investigators isolated 2-thio-5- (or 6-) carboxymethyluridine in the form of a methyl ester from yeast tRNA which had been hydrolyzed to its constituent nucleosides by a mixture of venom phosphodiesterase and bacterial phosphomonoesterase. In the course of the present study 5-carboxymethyluridine 5′-phosphate could not be found in a phosphodiesterase hydrolysate of tRNA, but since it could be recovered after alkali treatment of the 5′-nucleotides from the same hydrolysate, it is possible that 5-carboxymethyluridine may also occur as a carboxylate ester in native tRNA. Such an ester would be expected to appear as a nucleoside 5′-monophosphate in a phosphodiesterase hydrolysate of RNA, and since it would have a net charge of −2 at pH 7.8, this ester would elute from DEAE-cellulose *together with* the other 5′-nucleotides at pH 7.8. Alkali treatment of the isolated 5′-nucleotides would then lead to saponification of the ester, and upon rechromatography of the alkali-treated nucleotides, the resulting carboxylic acid, with a net charge of −3 at pH 7.8, would elute from DEAE-cellulose *after* the other 5′-nucleotides, as was found to be the case. Further work is in progress concerning the nature of the component from which 5-carboxymethyluridylate is derived during alkali hydrolysis of yeast and wheat embryo tRNA.

The isolation of methyl 2-thiouridine-5- (or 6-) acetate from yeast tRNA raises the question of whether the 5-carboxymethyluridine which we have isolated from yeast tRNA could be an alkali degradation product of 2-thio-5-carboxymethyluridine. This is considered unlikely for the following reasons. (1) The yield of methyl 2-thiouridine-5- (or 6-) acetate from yeast tRNA reported by Baczynskyj *et al.* (1968) is almost 100-fold smaller than the yield of 5-carboxymethyluridylate which we obtain from alkali hydrolysates of brewers' yeast tRNA; (2) treatment of ethyl 2-thiouracil-5-ace-

tate with aqueous potassium hydroxide has been reported to result in a quantitative yield of the corresponding acid, 2-thiouracil-5-acetic acid (Johnson, 1911). In addition, two sulfur-containing nucleotides, 4-thiouridylate and a 1-substituted 2-thiouracil derivative, have been isolated from alkali hydrolysates of *E. coli* tRNA (Lipsett, 1965; Carbon *et al.*, 1965). These observations suggest that 2- and 4-thiouracil derivatives are relatively stable to alkali, and are not converted into the corresponding keto derivatives under alkaline conditions.

All tRNA preparations from which 5-carboxymethyluridine was isolated were purified in order to specifically remove any contaminating low molecular weight nucleotides, although in fact there was little evidence of such contamination during purification of the yeast tRNA and wheat embryo tRNA used in this study. The isolation of 5-carboxymethyluridylate in highly reproducible amounts from alkali hydrolysates of various samples of yeast tRNA and wheat embryo tRNA argues against the possibility that this compound originates as (or from) a low molecular weight contaminant. The fact that after hydrolysis of tRNA we have been able to isolate 5-carboxymethyluridine in the form of its 2′-, 3′-, and 5′-phosphate isomers favors the view that this compound originally occurs as part of a ribonucleate chain, covalently bound in the usual 3′–5′ phosphodiester linkage.

The question of whether 5-carboxymethyluridine might have been derived from some polymer other than tRNA cannot be answered definitively at this time. There is evidence that small amounts of other natural polynucleotides are isolated along with tRNA during phenol extraction (Brown, 1963; Richards and Gratzer, 1964; Hindley, 1967), and at least some of these contaminant polymers might accompany tRNA throughout all the purification procedures employed, including salt fractionation. Based on their capacity to accept amino acids, our purified tRNA samples certainly contained more than 90% tRNA, but although these preparations were routinely chromatographed on DEAE-cellulose in order to remove any high molecular weight rRNA, the chromatographically purified yeast and wheat embryo tRNA may still have contained 5–10% of 5S RNA, judging from the quantity of uridine released from purified tRNA upon alkali hydrolysis (Hudson *et al.*, 1965; Gray and Lane, 1967). While 5S RNA itself does not contain minor components (Brownlee *et al.*, 1967; Forget and Weissman, 1967; Hindley, 1967), its probable presence in the extensively purified tRNA makes it conceivable that other non-tRNA polynucleotides could be present in trace amounts, and that 5-carboxymethyluridine might be present in one or more of these polymers. This same point was raised by Hall (1965) with regard to his isolation of 2′(3′)-*O*-ribosyladenosine from yeast "soluble" RNA, and recent evidence suggests that this particular compound is in fact a component of a natural polynucleotide other than tRNA (Hasegawa *et al.*, 1967). It should be pointed out, however, that if 5-carboxymethyluridine were in fact derived from a non-tRNA polynucleotide present in trace amounts in purified tRNA preparations, such a poly nucleotide would have to be composed entirely or in large part of 5-carboxymethyluridine residues, in order to account for the quantity of 5-carboxymethyluridylate isolated from alkali hydrolysates of yeast tRNA and wheat embryo tRNA. The question of whether 5-carboxymethyluridine is indeed a genuine constituent of tRNA will be most satisfactorily answered if it can be demonstrated that this compound is part of the primary structure of one or more chemically homogeneous RNAs.

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1-Methyladenosine. Dimroth Rearrangement and Reversible Reduction*

James B. Macon and Richard Wolfenden

ABSTRACT: Rearrangement of 1-methyladenosine to 6-methylaminopurine ribonucleoside proceeds at room temperature at a rate proportional to hydroxide ion concentration below pH 8 and above pH 10, with a plateau between. The results are consistent with hydroxide attack on both the protonated and neutral forms of the nucleoside, whose pK_a is 8.25. 1-Methyladenosine is rapidly reduced by sodium borohydride at pH 8.2 to 1-methyl-6-hydroadenosine, pK_a = 11.9. The reduced compound is oxidized by air in alkaline solution to 6-methylaminopurine ribonucleoside, and by nitrous acid at pH 5.4 to 1-methyladenosine. 3-Methylcytidine is also reduced by sodium borohydride, but deamination renders this reaction irreversible.

1-Methyladenine and 3-methylcytidine occur in small quantities in tRNA (Dunn, 1961; Hall, 1967). 1-Methyladenylic acid residues occur in known positions of the nucleotide sequence of yeast tyrosine (Madison *et al.*, 1967) and phenylalanine (RajBhandary *et al.*, 1966) tRNA corresponding to looped-out regions of the secondary structure.

These bases are unusual in being protonated at physiological pH values as discussed below. Modifications of these bases are therefore expected to influence electrostatic interactions in nucleic acids in which they occur, and may also be useful in structural studies. This paper describes two modifications of the free nucleosides, borohydride reduction and Dimroth rearrangement. Both modifications are found to occur at room temperature near neutrality. The product of borohydride reduction of adenosine can be reoxidized selec-

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