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.YEAST...AND..WHEAT..EMBRYO..TRANSFER RNA

UNIVERSITY....ALBERTA.....

DEGREE.....Ph.D.....YEAR GRANTED....1968.....

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(Signed)..Michael..W..Gray

PERMANENT ADDRESS:

..1034..Bassett Crescent,

.Medicine.Hat.,.Alberta,

...Canada.....

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5-Carboxymethyluridine, A Novel Nucleoside Derived from Yeast and Wheat Embryo Transfer RNA

by

© Michael William Gray

A Thesis

Submitted to the Faculty of Graduate Studies

In Partial Fulfilment of the Requirements for the Degree

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August, 1968

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "5-Carboxymethyluridine, a Novel Nucleoside Derived from Yeast and Wheat Embryo Transfer RNA", submitted by Michael William Gray in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

D. Lane
Supervisor

Cyril M. Kay

C. J. Smith

Neil Madsen

Asad Ahmad

Ron J. Hall
External Examiner

Date August 6, 1968

ABSTRACT

The present investigation was initiated with the aim of obtaining information about the structure of certain unidentified compounds present in chemical and enzymic hydrolysates of transfer RNA. As a result of this investigation, a novel nucleoside, 5-carboxymethyluridine, has been identified. This nucleoside appears to be a constituent of certain of the transfer RNA molecules in yeast and wheat embryo, and as such, it is the first carboxyl-containing minor component to be isolated from RNA.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. B. G. Lane for his unfailing counsel and encouragement throughout the course of this work.

I would like to thank Dr. C. M. Kay for use of the Spinco Model E ultracentrifuge, Dr. L. B. Smillie for use of the Beckman 120C amino acid analyzer, Dr. W. Bridger for use of the Cary 15 spectrophotometer, and Dr. P. G. Barton for use of the Hoover melting point apparatus.

The assistance of Mr. Morris Aarbo, who operated the ultracentrifuge, and of Mr. Alan Davidson, who operated the amino acid analyzer, is gratefully acknowledged. I am also grateful to the technical staff of the Department of Chemistry, University of Alberta, for determination of nuclear magnetic resonance, infrared, and mass spectra.

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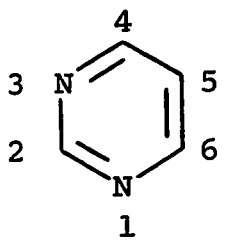
ABBREVIATIONS

n	unspecified purine or pyrimidine (a = adenine, c = cytosine, g = guanine, u = uracil)
N	unspecified ribonucleoside (A = adenosine, C = cytidine, G = guanosine, U = uridine)
Np	nucleoside 2'(3')-monophosphate
pN	nucleoside 5'-monophosphate
pNp	nucleoside 2'(3'),5'-bisphosphate
pN>p	nucleoside 2',3'(cyclic),5'-bisphosphate
Nm	O$^{2'}$-methylnucleoside
NmpNp	alkali-stable dinucleotide
NmpN	alkali-stable dinucleoside phosphate
Ψ	pseudouridine
UPRP	β-ureidopropionic acid N-ribosylphosphate
RNA	ribonucleic acid (ribonucleates)
DNA	deoxyribonucleic acid (deoxyribonucleates)
tRNA	transfer RNA
rRNA	ribosomal RNA
sRNA	that portion of the cellular RNA soluble in 1 M sodium chloride solution at 0° (mainly tRNA, but also containing small amounts of 5 → 7 S RNA from ribosomes)
DEAE-cellulose	O-(diethylaminoethyl) cellulose
DEAE-carbonate, DEAE-chloride, DEAE-formate	DEAE-cellulose in the carbonate, chloride, and formate counterion forms, respectively
PDE	snake venom phosphodiesterase
PME	E. coli alkaline phosphatase (a non-specific phosphatase)

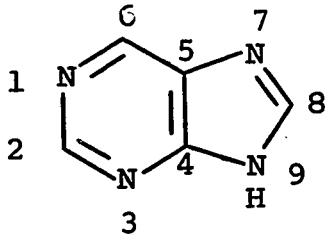
ATP	adenosine 5'-triphosphate
AMP	adenosine 5'-monophosphate
Tris	tris(hydroxymethyl) aminomethane
ir	infrared
nmr	nuclear magnetic resonance
uv	ultraviolet
M^+	molecular ion
S	Svedberg unit (1 S = 1 X 10^{-13} second)
conc.	concentrated

The system used to designate the methylated nucleoside components of RNA is as follows: The letter "m" is placed to the right of the nucleoside symbol if the component is methylated at the $\underline{O}^{2'}$-position of the ribose residue (e.g., Am = $\underline{O}^{2'}$-methyladenosine). If the component is methylated in the N-heterocycle (base-methylation), the letter "m" is placed to the left of the nucleoside symbol (e.g., mA = a base-methylated adenosine). To specify the position of base-methylation, a superscript is placed between the "m" and the nucleoside symbol (e.g., m^1A = 1-methyladenosine). If more than one methyl group occurs at a particular position, a subscript is placed between the "m" and the nucleoside symbol to specify the number of methyl groups (e.g., m_2^6A = $\underline{N}^6$,$\underline{N}^6$-dimethyladenosine). Other symbols used: "cm" = carboxymethyl- (e.g., cm^5U = 5-carboxymethyluridine); "h" = hydro- (e.g., h_2U = dihydrouridine)

The numbering of the purine and pyrimidine ring systems is as follows:



pyrimidine



purine

PREFACE

With the development of discerning analytical techniques during the past twenty years, knowledge of the structural chemistry and biological function of RNA has greatly increased. Prior to the early 1950's, RNA was a rather ill-defined substance. Ribonucleate preparations of this period were badly degraded and bore little resemblance to native RNA, since the susceptibility of ribonucleates to chemical and enzymic degradation was not generally appreciated. Much confusion as to the molecular size of native RNA resulted when physical and chemical studies were carried out on such degraded RNA specimens. The development of methods for the isolation of <u>undegraded</u> cellular RNA provided impetus for controlled chemical, physical, and biological studies of the cellular ribonucleates. It soon became apparent that the RNA within cells could be divided into several discrete morphological and functional classes, such as ribosomal, transfer, and messenger, and it was further recognized that the various functional classes of RNA contained macromolecules having unique covalent structures. As in the case of proteins, the realization that the various species of RNA were macromolecules of defined structure, rather than ill-defined "colloids", greatly stimulated research in ribonucleate chemistry and biology.

To complement the advances mentioned above, techniques were simultaneously developed for careful chemical analysis of RNA. The classical techniques of organic chemistry had allowed

TABLE I. MINOR NUCLEOSIDE COMPONENTS OF TRANSFER RNA

Derivatives of Adenosine	Derivatives of Cytidine	Derivatives of Guanosine	Derivatives of Uridine
1-methyladenosine	3-methylcytidine	1-methylguanosine	pseudouridine (5-ribosyl-uridine)
N^6-methyladenosine	5-methylcytidine	7-methylguanosine	dihydrouridine
N^6,N^6-dimethyladenosine	N^4-acetylcytidine	N^2-methylguanosine	3-methyluridine
N^6-(Δ^2-isopentenyl)-adenosine	$O^{2'}$-methylcytidine	N^2,N^2-dimethylguanosine	5-methyluridine
N^6-(*cis*-4-hydroxy-3-methyl-butenyl)adenosine		$O^{2'}$-methylguanosine	$O^{2'}$-methyluridine
2-methyladenosine			$O^{2'}$-methylpseudouridine
$O^{2'}$-methyladenosine			4-thiouridine
inosine			5-carboxymethyluridine
1-methylinosine			2-thiouridine-5-acetic acid methyl ester

the isolation and characterization of four heterocyclic nitrogenous bases (adenine, cytosine, guanine and uracil) from RNA, as well as their corresponding nucleosides (N,β,D-ribofuranosyls of the bases) and nucleotides (phosphate monoesters of the nucleosides). Further investigation demonstrated that the nucleotides are the fundamental structural units of RNA, and that ribonucleate molecules are poly-nucleotide chains in which the monomer nucleotides are linked to each other through 3' → 5' phosphodiester bridges. These latter studies were greatly aided by new analytical techniques such as paper chromatography, paper electrophoresis, and ion-exchange chromatography. Since the newer analytical techniques provided a degree of resolution unobtainable with the classical chemical techniques, minor components began to be encountered during analyses of chemical and enzymic hydrolysates of RNA. In most cases, it has been established that these minor components are part of the covalent structure of RNA, and that they are not simply artifacts formed during the isolation or subsequent hydrolysis of the RNA.

Of the various classes of cellular RNA, transfer RNA contains the widest variety and largest quantity of minor components. As illustrated in Table I, all of the minor components isolated to date can be considered to be chemical modifications of the four major nucleoside constituents (adenosine, cytidine, guanosine and uridine) that are found in all types of RNA. The modified portion of the nucleoside may be the base, the sugar, or the base-sugar bond. By virtue of their

varied chemical structures, the minor components can impart to transfer RNA certain unique properties, through which the biological function of transfer RNA might conceivably be regulated. Thus, in addition to the interest which the minor components have elicited as new natural products, they are also being actively studied in the hope that a knowledge of their chemistry may lead to an understanding of the relationship between the chemical structure of transfer RNA and its biological role as an intermediary in protein synthesis.

The work reported in this thesis is primarily concerned with the isolation and characterization of a new minor component of tRNA, 5-carboxymethyluridine. This nucleoside was discovered during studies of the $\underline{O}^{2'}$-methylnucleoside constituents of wheat embryo tRNA (Hudson, Gray and Lane, 1965) and yeast tRNA (Gray and Lane, 1967). Before the structure of 5-carboxymethyluridine was known, its properties seemed unusual enough to warrant a more detailed investigation, and the results of this investigation are reported in the second section (Part II) of the thesis.

Minor components may be isolated from tRNA at three "levels" of structural integrity, i.e., as nucleotides, as nucleosides, or as bases. Nucleosides are amenable to preliminary fractionation by column partition chromatography using organic solvents, and this method has been developed and extensively utilized by Hall for the preparation of many of the minor components of tRNA (see, for example, Hall, 1965). On the other hand,

nucleotides are readily fractionated by chromatography on DEAE-cellulose or Dowex anion-exchange resins, and this procedure was used in the present study for the isolation of the 2'-, 3'-, and 5'-nucleotides of 5-carboxymethyluridine. In addition to the nucleotides of 5-carboxymethyluridine, several other interesting compounds were encountered during the large-scale ion-exchange fractionation of hydrolysates of tRNA. The preliminary characterization of these unknown compounds is presented in Part III of the thesis.

In any study involving the isolation of minor components from tRNA, it is desirable that the tRNA be undegraded, highly-purified, and well-characterized, in order to minimize the possibility that the minor components arise from a contaminant of the tRNA. The first section (Part I) of the thesis contains a description of the methods used to maintain the integrity of tRNA during its isolation and to insure its subsequent purity. Some of the chemical and physical characteristics of the purified tRNA preparations are presented in Part I, along with a study of the amino acid-acceptor activity of wheat embryo tRNA.

I. ISOLATION, PURIFICATION, AND CHARACTERIZATION OF TRANSFER RNA

(1) Introduction

The most widely used method for the isolation of undegraded RNA from viruses, cells and tissues was introduced little more than ten years ago (Gierer and Schramm, 1956; Colter and Brown, 1956; Kirby, 1956). This method involves extraction with phenol in the presence of a low ionic strength buffer, followed by centrifugation to separate the extract into an upper, aqueous layer (containing RNA and polysaccharides), and a lower, phenol layer (containing protein). In addition to effecting a separation of RNA and protein, the method has the advantage that DNA is insoluble under the conditions of extraction, so that it is not necessary to carry out a preliminary separation of nuclei before beginning the isolation of RNA. Model experiments with pancreas ribonuclease have indicated that this nucleolytic enzyme is rendered inactive by its extraction into the phenol phase (from which it can be recovered fully active). It is reasonable to expect that other cellular nucleases would also be inactivated, either by extraction into the phenol phase or by denaturation. Finally, the entire isolation procedure is carried out near neutrality and at room temperature, avoiding the pH extremes and high temperatures known to cause degradation of ribonucleates.

After centrifugation of a phenol extract of whole yeast cells, the aqueous phase contains transfer RNA but no high

molecular weight ribosomal RNA (Monier, Stephenson and Zamecnik, 1960). The yeast cell wall apparently remains intact during phenol extraction and is only permeable to RNA of relatively low molecular weight (e.g., transfer RNA). After phenol extraction of wheat embryo, however, both ribosomal RNA and transfer RNA are present in the aqueous phase, and in fact the quantity of rRNA is several times greater than that of tRNA (Lane and Allen, 1961; Glitz and Dekker, 1963). Crestfield, Smith and Allen (1955) observed that high molecular weight ribosomal RNA is insoluble in aqueous solutions of elevated ionic strength, whereas tRNA is soluble under the same conditions. This differential solubility provides the basis for an effective separation of these two classes of RNA. Thus, when the aqueous, RNA-containing phase obtained after phenol extraction of wheat embryo is made 3 M with respect to sodium chloride, and allowed to remain at 0°, the rRNA forms a gel-like precipitate which can be removed by centrifugation, while the tRNA remains in solution[1].

---

[1]It was observed on several occasions in the course of the present investigation that when concentrated aqueous solutions of wheat embryo tRNA (ca. 15 mg/ml) were made 1 M with respect to sodium chloride, and allowed to stand at 0°, part of the tRNA crystallized in the form of thin plates. These crystals were soluble in water, and such solutions had ultraviolet absorption spectra typical of RNA. Moreover, the uv-absorbing material in these crystals chromatographed as tRNA on DEAE-cellulose.

After removal of rRNA, the tRNA is still contaminated with considerable quantities of polysaccharides, which can be eliminated by extracting the crude tRNA in a two-phase system composed of phosphate buffer and 2-methoxyethanol, as described by Kirby (1956). The tRNA is soluble in the organic phase, whereas the neutral and acidic polysaccharides are partly soluble in the aqueous phase, the remainder forming an insoluble layer between the aqueous and organic phases. 2-Methoxyethanol and salt can be removed by dialysis of the tRNA-containing organic phase, but a more convenient procedure for recovery of tRNA from the organic phase involves its precipitation in the form of a hexadecyltrimethylammonium salt (Ralph and Bellamy, 1964). Besides minimizing the risk of enzymic and chemical degradation inherent in the dialysis procedure, the precipitation method provides a better separation of tRNA from 5'-mono- and polyphosphates and other phosphate esters. These low molecular weight contaminants are incompletely removed from tRNA even after prolonged dialysis, but they remain in the organic phase during the precipitation of tRNA as its CTA salt.

Conditions for storage of purified ribonucleate preparations should be chosen with a view to minimizing thermally-induced hydrolytic cleavage of phosphodiester bonds (Diemer, McLennan and Lane, 1966). Removal of water from RNA precipitated from 67% ethanol may be accomplished by successive washing with ethanol and ether, followed by air-drying of the washed RNA. In the case of tRNA, it has been found that the

relatively anhydrous powder obtained by this procedure can be stored at -20° for several years without apparent degradation. Since it has been shown that magnesium ions markedly increase the rate of spontaneous hydrolysis of ribonucleates (Diemer, 1965), all RNA preparations were stored in the sodium counterion form.

(2) Preparation of Wheat Embryo Transfer RNA

Freshly-milled wheat embryo was stored in a cold room (near 0°) until used. Portions of wheat embryo (125 g) were suspended in 500 ml 0.05 M potassium phosphate buffer (equimolar in KH_2PO_4 and K_2HPO_4; pH ca. 7) before mixing with 500 ml water-saturated (approx. 90%) phenol. The mixtures, in thick-walled glass containers, were then agitated on a horizontal shaker for 20 min at room temperature, after which the contents of each container were transferred to 1 l. polyethylene bottles and centrifuged for 15 min at 2000 rpm (International Portable Refrigerated Centrifuge Model PR-2; rotor 276) The temperature of the centrifuge was maintained at ca. 5° (lower temperatures may induce crystallization of the phenol). The upper aqueous layers were carefully withdrawn by vacuum suction into a flask cooled in ice; care was taken to avoid the insoluble material at the interphase. A total of 3 kg of wheat embryo was processed in this manner, yielding 5 - 6 l. of RNA-containing solution, which was filtered through glass wool to

remove a small amount of insoluble material. Solid sodium chloride was added to the filtrate to a final concentration of 3 M, and after thorough mixing, the resulting solution was placed in the cold.

The rRNA formed a gel-like precipitate upon standing overnight at 0° in the presence of the high concentration of salt, while some of the phenol contained in the solution "salted-out" in the form of fine crystals. During centrifugation of the resulting suspension, a portion of the rRNA sedimented, while the remainder associated with the phenol crystals, forming a buoyant "complex" which floated on top of the tRNA-containing supernatant. This "complex" was removed by vacuum suction, and the underlying supernatant was carefully decanted from the rRNA pellet. Re-centrifugation of the phenol-rRNA "complex" caused most of the rRNA to sediment; the tRNA-containing supernatant was decanted after an overlying layer of phenol crystals was removed. The combined tRNA-containing supernatants were filtered through glass wool to remove traces of insoluble rRNA, and the tRNA was precipitated by addition of two volumes 95% ethanol. The suspension was then placed in the cold room (0°).

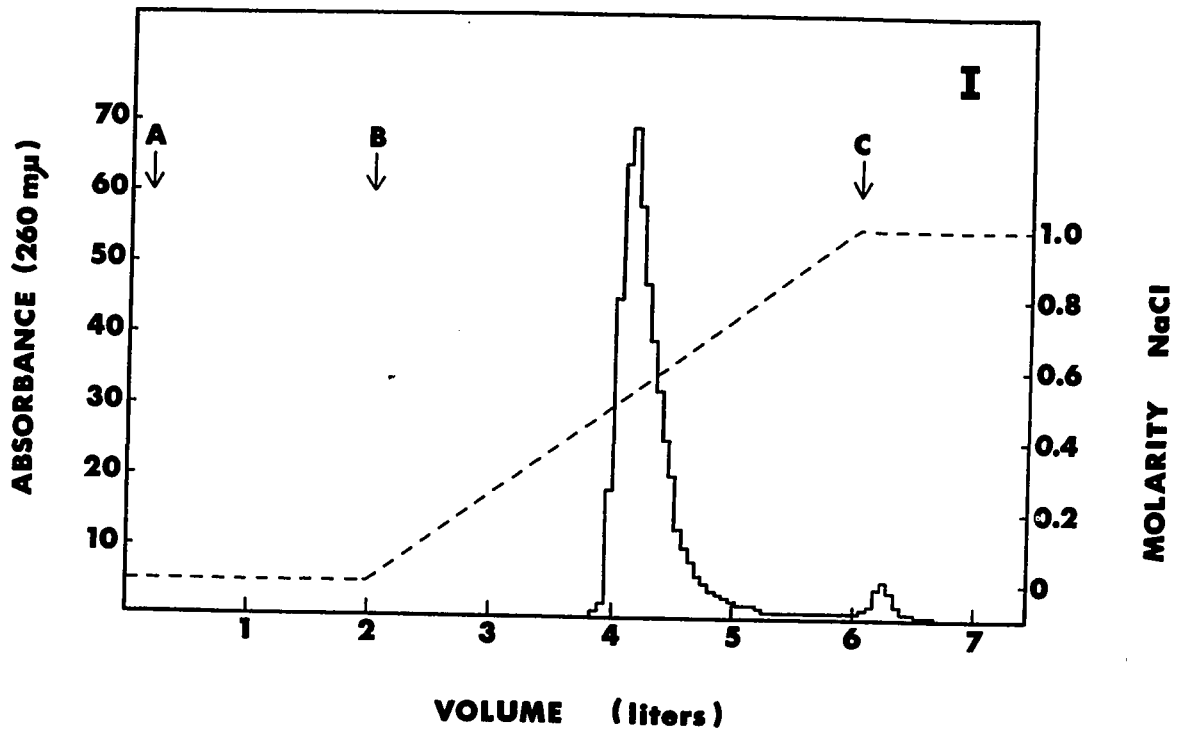
The precipitated tRNA (and polysaccharides) readily settled overnight, so that the bulk of the overlying liquid could be removed by vacuum suction. The remainder of the suspension was distributed among four 1 l. polyethylene bottles, and the crude tRNA was collected by centrifugation. Each pellet was washed twice with 500 ml cold 67% ethanol, in order

to remove residual phenol. The washed pellets were then dissolved in cold water, and the resulting opalescent solution was passed through a coarse sintered glass filter in order to remove any coarse insoluble material. After adjusting the volume of the filtrate to 2 l., aliquots (250 ml) of the opalescent tRNA solution were added to a mixture of 250 ml 2.5 M K_2HPO_4 and 12.5 ml 33.3% H_3PO_4 in a heavy-walled glass bottle (pH of the K_2HPO_4-H_3PO_4 mixture was ca. 8). 2-Methoxyethanol (250 ml) was added, resulting in an immediate precipitation of polysaccharides. Each suspension was extracted on a horizontal shaker for 5 min at room temperature, then immediately transferred to 1 l. polyethylene bottles and centrifuged (2000 rpm, 15 min, 5°). The upper (organic) phases were removed by vacuum suction into a cooled flask; care was taken to avoid the insoluble polysaccharides at the interphase. One-half volume cold water and one-half volume 1% cetyltrimethylammonium bromide (CTA-Br) were then added to the combined organic phases. Precipitation of CTA-tRNA occurred immediately, and the suspension was then left at 0° overnight. The insoluble CTA-tRNA was collected by centrifugation, and the precipitate was divided into two equal parts. Each part was washed with three 500 ml portions of cold water, in order to remove residual 2-methoxyethanol, phosphate buffer, and CTA-Br. The CTA-tRNA was then converted to the sodium salt by washing each of the two pellets with three 500 ml portions of cold 80% ethanol, 0.1 M in sodium acetate; CTA-acetate was removed in the ethanol supernatant, whereas Na-tRNA remained insoluble.

The sodium transfer ribonucleates were dissolved in ca. 200 ml water, and the resulting solution was passed through a medium sintered glass filter. The filtrate was transferred to a 1 l. polyethylene bottle, the volume was adjusted to ca. 250 ml, and solid sodium acetate was added to a final concentration of 0.1 M. Two volumes of cold 95% ethanol were then added to precipitate the tRNA, and after 1 hr at 0°, the precipitate was collected by centrifugation. The supernatant was carefully decanted and the pellet re-dissolved in 250 ml cold water and re-precipitated as above. This repeated precipitation of tRNA in the presence of sodium acetate was carried out in order to remove contaminating anions such as chloride and phosphate, and cations such as magnesium.

After the final precipitation, the tRNA pellet was washed in succession with four 500 ml portions of each of the following solutions: 95% ethanol-diethyl ether-5% sodium acetate (3:1:1, by vol.); 95% ethanol-diethyl ether (1:1, by vol.); and finally, anhydrous diethyl ether. The residual ether was allowed to evaporate at room temperature. The tRNA was constantly stirred during evaporation of the ether, and it quickly dried to a fine white powder. The anhydrous powder was then stored in a tightly-closed container at -20°. The final yield from 3 kg of wheat embryo was 5 - 6 g of tRNA (0.17 - 0.20 wt. %). A 1% aqueous solution of wheat embryo tRNA prepared by the above procedure had an extinction ranging from 185 to 200 at 260 mμ (1 cm cell). Assuming that pure, completely anhydrous tRNA has an extinction of 250 under the same conditions, this

FIGURE 1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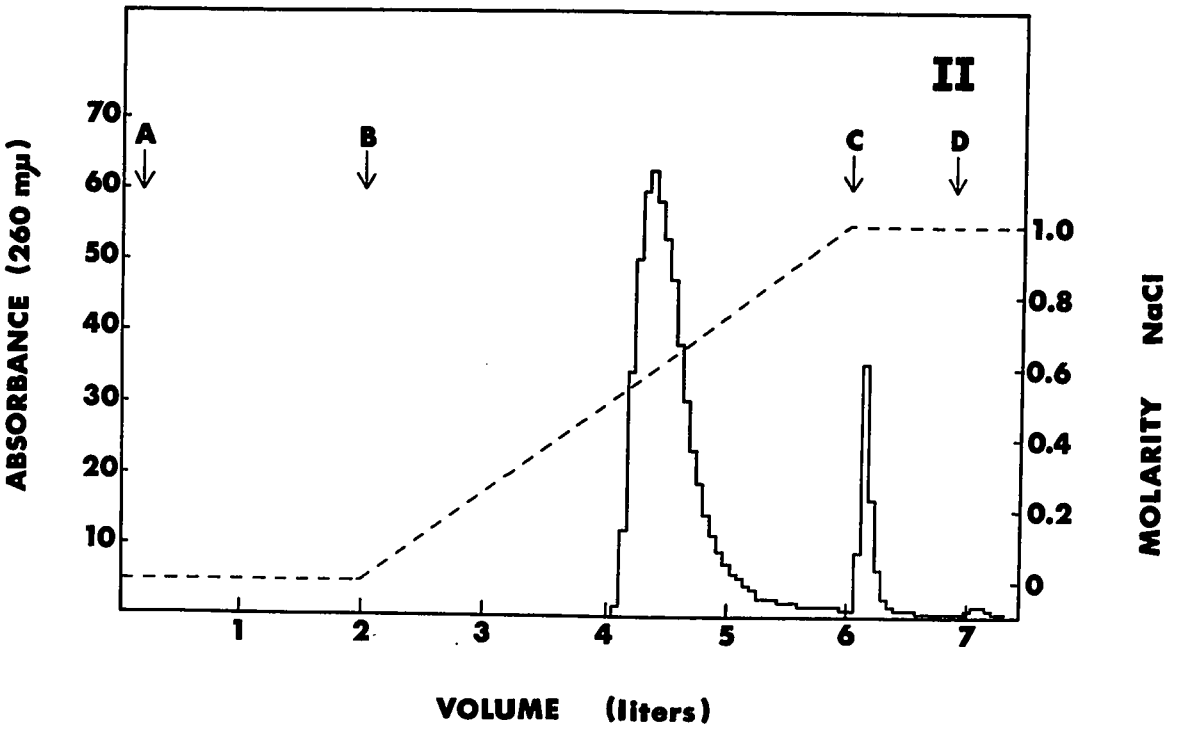
ABSORBANCE (260 mμ)
MOLARITY NaCl
VOLUME (liters)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ABSORBANCE (260 mμ)
MOLARITY NaCl
VOLUME (liters)

LEGEND OF FIGURE 1

Diagrams illustrating the elution profiles observed during DEAE-cellulose chromatography of commercial preparations of brewers' yeast tRNA (I) and *E. coli* B tRNA (II). Details of the chromatography are given in the text. The amount of uv-absorbing material applied to the column was 30,300 A_{260} units in the case of the yeast tRNA, and 39,250 A_{260} units in the case of the *E. coli* tRNA. The eluents used, as indicated by the arrows, were: (A) 0.1 M Tris chloride (pH 7.6); (B) 4 l. gradient from 0 to 1.0 M sodium chloride, 0.1 M in Tris chloride (pH 7.6); (C) 1.0 M sodium chloride adjusted to pH 11.0 with ammonia; (D) 1.0 M sodium chloride-0.1 M sodium hydroxide.

represents a minimum purity of 75 - 80%.

(3) Purification of Yeast and E. coli Transfer RNA

The yeast and E. coli transfer RNA preparations used in this study were purchased from commercial firms. Bakers' yeast tRNA and E. coli B tRNA were obtained from General Biochemicals, while brewers' yeast tRNA was a product of Boehringer Mannheim Corp. These preparations, as well as the wheat embryo tRNA prepared as described in the preceding section, were purified by gradient elution chromatography on DEAE-cellulose (see, for example, Glitz and Dekker, 1963; Bell, Tomlinson and Tener, 1964). Solutions of the tRNA samples were analyzed in the ultracentrifuge (Spinco Model E) before chromatography, and all preparations, except the E. coli tRNA, showed a single symmetrical peak with a sedimentation coefficient of ca. 4 S (determined for a 0.3% solution of tRNA in 0.15 M sodium chloride). In the case of the E. coli tRNA, a small peak sedimenting faster than the main tRNA peak indicated a slight contamination with larger molecular weight material, e.g., ribosomal RNA or DNA.

Figure 1 shows the elution profiles observed during DEAE-cellulose chromatography of commercial preparations of brewers' yeast tRNA (I) and E. coli tRNA (II). The tRNA (ca. 2 g) was dissolved in 100 ml 0.1 M Tris chloride (pH 7.6), and the solution obtained was applied to a column of DEAE-chloride,

4.25 cm (i.d.) X 20 cm (h.). The column was washed with 2 l. 0.1 M Tris chloride (pH 7.6), after which a linear gradient from 0 to 1.0 M sodium chloride in 0.1 M Tris chloride (pH 7.6) was started; the total volume of the gradient was 4 l. A flow rate of 12 ml/min was maintained, and 60 ml fractions were collected. Under these conditions, a large uv-absorbing peak corresponding to tRNA began to appear when the sodium chloride concentration of the eluent reached 0.5 M. No significant uv-absorbing material was eluted before the tRNA, indicating the absence of low-molecular weight nucleotides (such as ATP) in these particular preparations. Elevation of the pH of the eluent after elution of the tRNA resulted in the appearance of further uv-absorbing material, particularly in the case of the E. coli tRNA. This material most likely corresponds to contaminating ribosomal RNA, which remains firmly bound to DEAE-cellulose during elution with salt gradients (Monier, Stephenson and Zamecnik, 1960). The E. coli tRNA which eluted from DEAE-cellulose accounted for 80% of the uv-absorbing material applied to the column, while in the case of the brewers' yeast tRNA, 98% of the uv-absorbing material applied to the column was recovered in the eluted tRNA peak. The chromatographic behaviour of the preparations of bakers' yeast tRNA and wheat embryo tRNA paralleled the behaviour of the brewers' yeast tRNA on DEAE-cellulose, in that contaminant low-molecular weight nucleotides were absent, while essentially all of the applied uv-absorbing material could be recovered in the tRNA peak eluted by the salt gradient.

Fractions containing transfer RNA were pooled, and the tRNA was precipitated by addition of two volumes of 95% ethanol. The precipitate was collected, washed and stored as described in the preceding section dealing with the preparation of wheat embryo tRNA. Aqueous solutions of purified bakers' yeast, brewers' yeast, and E. coli tRNA had $E^{1\%}_{1\ cm}$ values (at 260 mμ) of 185, 214, and 189, respectively.

(4) Physical and Chemical Characterization of Purified Transfer RNA

Sedimentation velocity analyses for the various purified preparations of yeast, wheat embryo, and E. coli tRNA were performed in a Spinco Model E ultracentrifuge employing a schlieren optical system. The sedimentation coefficients were measured at a ribonucleate concentration of 0.3%, in 0.15 M sodium chloride. In all cases, a single symmetrical peak was observed, and $s_{20,w}$ values ranged from 3.8 to 4.2 S. The sedimenting boundaries were homogeneous and material sedimenting faster or slower than the main tRNA peak was not detectable. Kay and Oikawa (1966) have carried out a detailed study of the hydrodynamic and optical rotatory dispersion properties of wheat embryo tRNA, isolated and purified by the methods just outlined.

Analyses of the terminal groups released by alkali hydrolysis of purified wheat embryo and yeast tRNA have been

TABLE II. END GROUPS ISOLATED FROM ALKALI HYDROLYSATES OF YEAST tRNA*

(1) Nucleosides (N)

	Bakers' Yeast tRNA	Brewers' Yeast tRNA
A	0.18 (11.8)	1.17 (92.1)
C	1.18 (77.1)	0.02 (1.6)
G	0.02 (1.2)	0.01 (0.8)
U	0.15 (9.9)	0.07 (5.5)
	1.53	1.27

(2) Nucleoside 2'(3'),5'-bisphosphates (pNp)

	Bakers' Yeast tRNA	Brewers' Yeast tRNA
pAp	0.039 (2.8)	0.042 (3.1)
pCp	0.039 (2.8)	0.041 (3.0)
pGp	1.11 (79.5)	1.07 (79.0)
pUp	0.15 (10.6)	0.17 (12.4)
pψp	0.060 (4.3)	0.034 (2.5)
	1.40	1.36

*The nucleosides found in alkali hydrolysates of yeast tRNA are derived from the 5'-linked termini of ribonucleate chains, while the nucleoside 2'(3'),5'-bisphosphates are derived from the 3'-linked termini. The details of these particular analyses have been published (Gray and Lane, 1967). The absolute amounts of nucleosides and nucleoside 2'(3'),5'-bisphosphates are given as moles of a particular end group per 100 moles of total nucleotides in an alkali hydrolysate. For each tRNA sample, the total quantity of nucleosides or nucleoside 2'(3'),5'-bisphosphates can be used to calculate the average chain length of the preparation, e.g., 1.36 moles/100 moles corresponds to a chain length of 74 (i.e., 100 ÷ 1.36). The relative amounts of nucleosides and nucleoside 2'(3'), 5'-bisphosphates (figures in brackets, above) give the percentage distribution of the individual end groups.

published (Hudson, Gray and Lane, 1965; Gray and Lane, 1967). Table II gives the proportions of the terminal groups isolated as nucleosides and nucleoside bisphosphates from alkali hydrolysates of bakers' and brewers' yeast tRNA. The terminal groups of the brewers' yeast tRNA were typical of those derived by alkali hydrolysis of tRNA from other sources, in that adenosine was the major nucleoside and guanosine 2'(3'),5'-bisphosphate was the major nucleoside bisphosphate. This situation was also true for the purified preparations of wheat embryo tRNA and E. coli tRNA. Although cytidine was the predominant nucleoside in alkali hydrolysates of bakers' yeast tRNA, this finding was not surprising in view of the fact that other workers have also noted that tRNA isolated from bakers' yeast is often deficient in the adenosine terminal (Ingram and Sjöquist, 1963; Bell, Tomlinson and Tener, 1964). The quantity of uridine released from purified yeast tRNA upon alkali hydrolysis most likely indicates the presence of 5 - 10% 5 S ribosomal RNA, which has been shown to terminate in uridine at the 5'-linked terminus and which, along with tRNA, is extracted from whole yeast cells by phenol (Hindley, 1967). Because it resembles transfer RNA in chain length (120 nucleotides vs. 80 nucleotides), and probably also in degree of secondary structure (Boedtker and Kelling, 1967), 5 S rRNA might be expected to accompany tRNA throughout the various purification procedures, including chromatography on DEAE-cellulose. The presence of 5 - 10% 5 S rRNA in the purified yeast tRNA preparations would not have been detected by analytical ultracentrifugation because of the

similarity in the sedimentation coefficients of the two types of RNA.

Yeast tRNA is notable in that pseudouridine is present at the phosphorylated end of 2 - 4 % of the chains. This terminal pseudouridine has been isolated as pseudouridine 2'(3'),5'-bis-phosphate from alkali hydrolysates of yeast tRNA (Gray and Lane, 1967), but none has so far been detected in alkali hydrolysates of wheat embryo or E. coli tRNA.

The results of analyses to determine the quantitative proportions of both the major and minor nucleotide constituents of transfer RNA are presented later in the thesis (Part III).

(5) Biological Activity of Wheat Embryo Transfer RNA

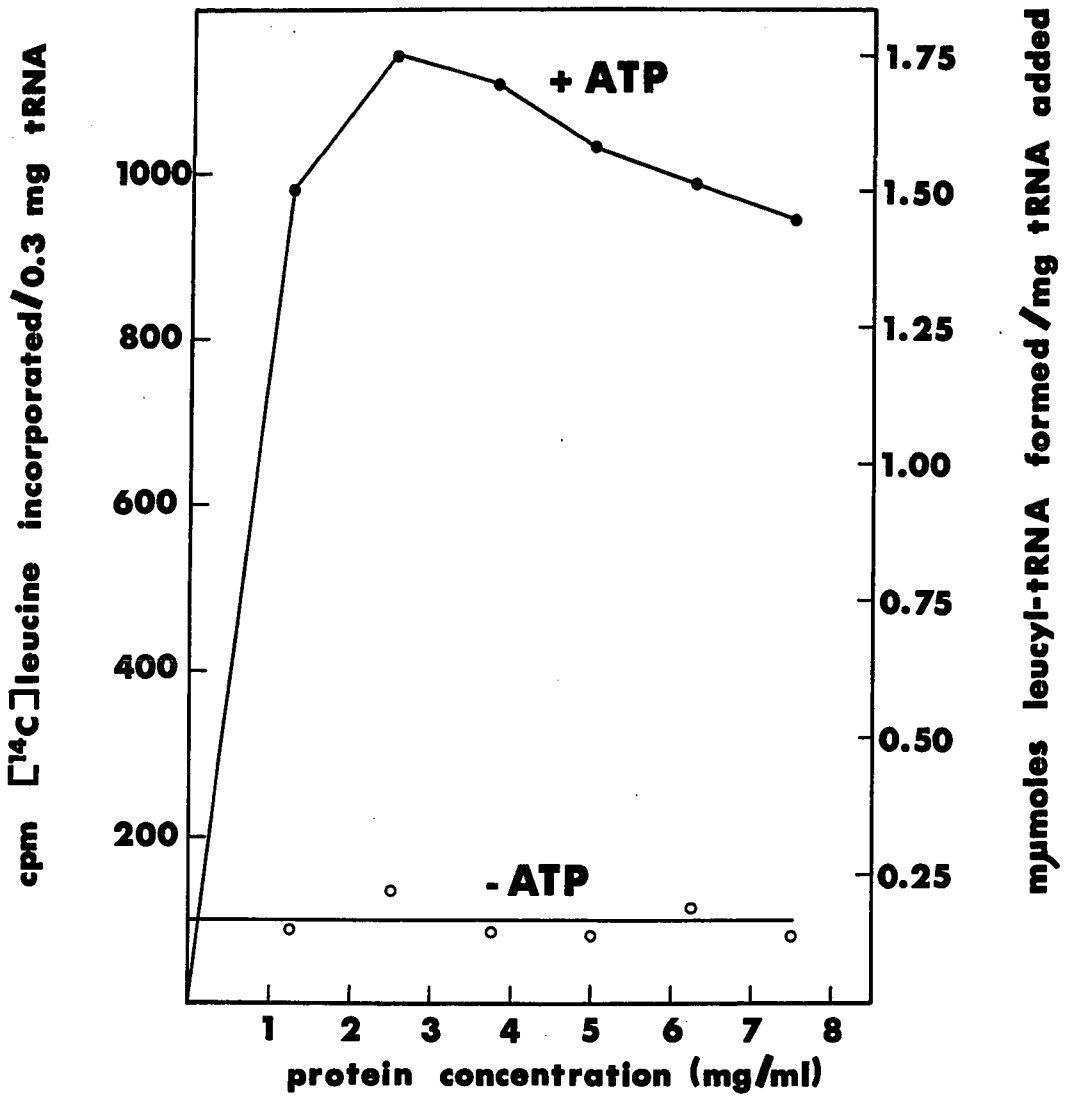
The biological activity of transfer RNA, as measured by its capacity to accept activated amino acids, is often a useful additional criterion for assessing the integrity and purity of tRNA preparations, and for this reason the amino acid acceptor capacity of purified wheat embryo tRNA was assayed.

A crude preparation of wheat embryo aminoacyl-tRNA synthetases (amino acid:tRNA ligases (AMP), EC 6.1.1.) was obtained by a modification of the method of Moustafa and Lyttleton (1963). Wheat embryo (120 g) was suspended in 200 ml cold phosphate-sucrose buffer (0.01 M potassium phosphate (pH 7.5), 0.4 M sucrose) in a Servall homogenizer. The mixture was maintained

near 0° (ice-water bath) and was ground at moderate speed (ca. 5000 rpm for 30 min) to a thick paste. The paste was then centrifuged at 500 X g (30 min at 0°), and the solid residue discarded. The supernatant solution was re-centrifuged at 20,000 X g (30 min at 0°), and the top creamy layer and a small amount of solid residue were discarded. Dialysis of the middle layer against 500 ml phosphate-sucrose buffer for 18 hr at 4° resulted in the formation of a heavy precipitate, which was removed by centrifugation at 39,000 X g (30 min at 0°). The relatively clear, yellow-brown supernatant solution was then re-centrifuged at 100,000 X g (90 min at 0°), in order to sediment ribosomes. A small amount of overlying insoluble material was removed, and about two-thirds of the supernatant solution in each tube was then carefully withdrawn. The combined supernatant solutions were dialyzed against 500 ml phosphate-sucrose buffer for 18 hr at 0°, resulting in removal of some of the yellow pigments contaminating the extract. The final clear solution (ca. 45 ml) was divided into 1 ml aliquots which could be stored at -20° with no detectable loss in activity for several weeks.

The final preparation of aminoacyl-tRNA synthetases had a protein concentration of ca. 60 mg/ml. For use in the assays, this stock solution was diluted with varying amounts of the phosphate-sucrose buffer, to a minimum protein concentration of 10 mg/ml. Storage of the diluted enzyme preparations at 0° (unfrozen) for several days resulted in no significant decrease in activity.

FIGURE 2


cpm [14C]leucine incorporated/0.3 mg tRNA
1000
800
600
400
200
+ ATP
- ATP
1.75
1.50
1.25
1.00
0.75
0.50
0.25
mμmoles leucyl-tRNA formed/mg tRNA added
1
2
3
4
5
6
7
8
protein concentration (mg/ml)

LEGEND OF FIGURE 2

A diagram illustrating the enzyme-catalyzed incorporation of [^{14}C]leucine into wheat embryo tRNA, as a function of the amount of crude wheat embryo supernatant (containing aminoacyl-tRNA synthetases) added. Reaction mixtures consisted of: 0.25 M Tris chloride (pH 7.5) [0.1 ml]; 0.1 M ATP (disodium salt, adjusted to pH 7.0 with NaOH) [0.1 ml]; 0.15 M $MgCl_2$ [0.1 ml]; [^{14}C]leucine, 3 μmoles/ml, sp. act. 1 μC/μmole [0.1 ml]; wheat embryo tRNA (sodium salt), 3 mg/ml [0.1 ml]; water [0.2 ml]. Controls lacked ATP. The reaction was started by addition of 0.1 ml of solutions of wheat embryo aminoacyl-tRNA synthetases of varying protein concentrations (10 - 60 mg/ml). After incubation at 37° for 20 minutes, cold 0.8 M $HClO_4$ (0.8 ml) was added to each mixture (ATP was added simultaneously to the controls). The precipitates were washed once with 2 ml cold 0.2 M $HClO_4$ and once with cold 95% ethanol, and then left to dry overnight at room temperature. The residues were dissolved in 0.6 ml conc. formic acid, and 500 μl aliquots of each solution were then transferred to copper planchets for determination of radioactivity.

The enzyme-catalyzed aminoacylation of wheat embryo tRNA was monitored by following the incorporation of [^{14}C] amino acids into perchloric acid-insoluble material. Assuming complete aminoacylation of tRNA in a reaction mixture containing unfractionated tRNA and all of the common amino acids, the theoretical yield of aminoacyl-tRNA is 1 mole per mole of uncharged tRNA added. Values in the literature are often expressed as "mμ-moles aminoacyl-tRNA formed per mg tRNA added", and a theoretical yield of 1 mole aminoacyl-tRNA/mole of tRNA added corresponds to ca. 36 mμmoles amino acid incorporated/mg tRNA, assuming the molecular weight of wheat embryo tRNA to be ca. 28,000. If a chemically homogeneous species of tRNA were being charged with its corresponding amino acid, a theoretical yield of 36 mμmoles/mg would also be expected, but in the case of unfractionated tRNA and only a single amino acid, a theoretical yield of the order of 1 - 2 mμmoles/mg is expected, since the tRNA specific for the amino acid in question comprises only a few per cent of the total tRNA added.

The experimental conditions used for the aminoacylation of wheat embryo tRNA varied somewhat during the course of this work, and so the exact conditions for each experiment are described in detail in the legends of the various figures and tables. In all cases, reactions were terminated by the addition of cold 0.8 M perchloric acid, which precipitated protein, tRNA, and aminoacyl-tRNA. In order to remove free [^{14}C] amino acids, the precipitates were washed successively with cold 0.2 M

TABLE III. AMINO ACID ACCEPTOR ACTIVITY OF WHEAT EMBRYO TRANSFER RNA

Amino Acid	[^{14}C] incorporated into $HClO_4$-insoluble material (cpm)			mμmoles aminoacyl-tRNA formed/mg tRNA added
	+ATP	-ATP	Net	
alanine	893	67	826	0.42
glutamic acid	1128	82	1046	0.53
leucine	1315	86	1229	0.62
lysine	1219	216	1003	0.51
serine	1454	86	1368	0.69
valine	1387	72	1315	0.66

Reaction mixtures consisted of: 0.25 M Tris chloride (pH 7.5) [0.1 ml]; 0.1 M ATP (disodium salt, adjusted to pH 7.0 with NaOH) [0.1 ml]; 0.15 M $MgCl_2$ [0.1 ml]; [^{14}C]amino acid, 3 μC/ml, 0.0125 - 0.04 μmole/ml [0.1 ml]; unlabelled amino acid, ca. 1 μmole/ml [0.1 ml]; wheat embryo tRNA (sodium salt), 3 mg/ml [0.1 ml]; water [0.1 ml]. A total of 0.1 μmole amino acid (labelled + unlabelled) was added in each case. Controls lacked ATP. The reactions were started by addition of 0.1 ml of a solution of wheat embryo aminoacyl-tRNA synthetases (20 mg protein/ml) to each mixture. After incubation at 37° for 20 minutes, cold 0.8 M $HClO_4$ (0.8 ml) was added to each mixture (ATP was added simultaneously to the controls). The precipitates were washed once with 2 ml cold 0.2 M $HClO_4$, once with 2 ml cold 95% ethanol, and then dried. The residues were dissolved in 0.6 ml conc. formic acid, and 500 μl aliquots of each solution were then transferred to copper planchets for determination of radioactivity.

perchloric acid and cold 95% ethanol, after which the washed precipitates were dried. Each residue was dissolved in conc. formic acid, and the resulting solutions (or aliquots of them) were transferred to copper planchets for determination of radioactivity using a Nuclear Chicago Model 186 gas flow counter (counting efficiency for ^{14}C at an operating voltage of 1400 was ca. 25%).

The effect of enzyme concentration on the incorporation of [^{14}C]leucine into perchloric acid-insoluble material is shown in Figure 2. Maximum formation of leucyl-tRNA was observed at a protein concentration of ca. 2 mg/ml, with the extent of formation steadily decreasing at higher protein concentrations. Similar results were obtained by Moustafa and Lyttleton (1963) for the incorporation of [^{14}C]valine into tRNA in the wheat embryo system. These workers attributed the decrease in extent of formation of valyl-tRNA at higher protein concentrations to the increasing hydrolysis of ATP by a non-specific pyrophosphatase contaminating the wheat embryo aminoacyl-tRNA synthetases. They found that while in simple buffer extracts of wheat embryo, the pyrophosphatase was present at a level which rapidly hydrolyzed the ATP used in their studies of amino acid activation, the addition of sucrose to the extraction medium (to minimize osmotic damage to the mitochondria) reduced the contaminating enzyme below the level where it interfered with amino acid activation. In the present study, assays were performed at a protein concentration which gave

TABLE IV. ACCEPTOR ACTIVITY OF WHEAT EMBRYO tRNA FOR SINGLE AMINO ACIDS AND FOR A MIXTURE

Amino Acid	[^{14}C] incorporated into $HClO_4$-insoluble material (cpm)			mμmoles aminoacyl-tRNA formed/mg tRNA added
	+Enzyme	-Enzyme	Net	
leucine	2240	2	2238	1.13
serine	2176	5	2171	1.10
valine	1445	17	1428	0.72
			5837	2.95
leucine + serine + valine	5113	22	5091	2.57

Reaction mixtures consisted of: 0.25 M Tris chloride (pH 7.5) [0.1 ml]; 0.1 M ATP (disodium salt dissolved in 0.2 M Tris; final pH = 7.5) [0.1 ml]; 0.1 M $MgCl_2$ [0.15 ml]; [^{14}C]amino acid (Leu, Ser, or Val), 1 μmole/ml, 3 μC/ml [0.1 ml]; wheat embryo tRNA, 3 mg/ml [0.1 ml]; water [0.35 ml]. In the case where all three amino acids were present in the same reaction mixture, 0.1 ml of each [^{14}C]amino acid solution was added, and the amount of added water was reduced to 0.15 ml. The reactions were started by addition of 0.1 ml of a solution of wheat embryo aminoacyl-tRNA synthetases (10 mg protein/ml) to each mixture. Controls lacked enzyme. After incubation at 37° for 20 minutes, cold 0.8 M $HClO_4$ (1 ml) was added to each mixture (enzyme was added simultaneously to the controls). The precipitates were washed three times with cold 0.2 M $HClO_4$ and once with cold 95% ethanol, and then dried for several minutes in a 250° oven. The residues were dissolved in 1 ml conc. formic acid, and 500 μl aliquots of each solution were then transferred to copper planchets for determination of radioactivity.

maximal formation of aminoacyl-tRNA; this optimal concentration was determined for each different preparation of aminoacyl-tRNA synthetases used.

Table III shows the results of the enzyme-catalyzed incorporation of several different amino acids into wheat embryo tRNA. It can be seen that the wheat embryo system displayed good activity for each of the six amino acids tested, although it appears that maximal formation of aminoacyl-tRNA was not attained in this particular experiment. The yield of leucyl-tRNA, for example, was only 0.62 mμmoles/mg, while using the same enzyme preparation, and at the same enzyme concentration (2.5 mg protein/ml), a yield of 1.73 mμmoles leucyl-tRNA/mg tRNA was obtained in a separate experiment (Figure 2). The higher incorporation of [^{14}C]leucine, however, was obtained at a leucine concentration of 0.375 μmoles/ml (Figure 2), which was three-fold greater than the leucine concentration employed in the assays described in Table III.

As shown by the data in Table IV, the incorporation of a given amino acid into its specific tRNA proceeds essentially independently of the incorporation of other amino acids into their specific tRNA's. When more than one amino acid is present in an aminoacylation mixture containing unfractionated tRNA, the total incorporation of label is approximately equal to the sum of the individual incorporations determined separately for each of the amino acids

TABLE V. INCORPORATION OF [^{14}C]LEUCINE INTO PRE-INCUBATED AND UNTREATED WHEAT EMBRYO tRNA

	[^{14}C] incorporated into $HClO_4$-insoluble material (cpm)			mμmoles aminoacyl-tRNA formed/mg tRNA added	
	+Enzyme	-Enzyme	Net		
Pre-incubated tRNA					
1	981	7	974	0.49)	0.52
2	1061	0	1061	0.54)	
Untreated tRNA					
1	1056	9	1047	0.53)	0.52
2	1027	0	1027	0.52)	
No tRNA	46	7	39	0.02	

Three mg wheat embryo tRNA were dissolved in 1 ml 1 M Tris chloride (pH 8.0), and the resulting solution was incubated at 37° for 90 minutes ("pre-incubated tRNA"). A second 3 mg sample of the same wheat embryo tRNA was dissolved in 1 ml water and placed at 0° (unfrozen) for 90 minutes ("untreated tRNA"). Duplicate aliquots [0.1 ml] of each of these tRNA solutions were then added to reaction mixtures consisting of: 0.1 M ATP (disodium salt) [0.1 ml]; 0.1 M $MgCl_2$ [0.1 ml]; [^{14}C]leucine, 1 μmole/ml, sp. act. 3 μC/μmole [0.1 ml]. 1 M Tris chloride (pH 8.0) [0.1 ml] had also been added to the reaction mixtures containing untreated tRNA. The total volume of each mixture was adjusted to 0.9 ml with water. A fifth reaction mixture contained Tris chloride, ATP, $MgCl_2$, and [^{14}C]leucine, but no tRNA. The reactions were started by addition of 0.1 ml of a solution of wheat embryo aminoacyl-tRNA synthetases (10 mg protein/ml) to each mixture. Controls lacked enzyme. After incubation at 37° for 20 minutes, cold 0.8 M $HClO_4$ (1 ml) was added to each mixture (enzyme was added simultaneously to the controls). The precipitates were washed three times with cold 0.2 M $HClO_4$ and once with cold 95% ethanol, and then dried for several minutes in a 250° oven. The residues were dissolved in 1 ml conc. formic acid, and 500 μl aliquots of each solution were then transferred to copper planchets for determination of radioactivity.

comprising the mixture.

In order to measure the extent of aminoacylation in freshly-isolated wheat embryo transfer RNA, the tRNA was treated under conditions similar to those which are known to effect hydrolysis of aminoacyl-tRNA linkages (Sarin and Zamecnik, 1964). The leucine-accepting capacity of wheat embryo tRNA which had been pre-incubated in 1 M Tris chloride (pH 8.0) at 37° for 90 minutes was almost exactly the same as the leucine-accepting capacity of untreated wheat embryo tRNA (Table V). This result suggests that essentially all of the leucine-tRNA, as isolated, is in the uncharged form*. The incorporation of [^{14}C]leucine in the absence of any added tRNA was only 4% of the incorporation observed when tRNA was present, indicating that negligible leucine-acceptor capacity was contributed by the crude enzyme preparation.

The foregoing results indicate that, in the presence of a crude preparation of wheat embryo aminoacyl-tRNA synthetases, wheat embryo transfer RNA isolated by the procedure

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*It should be noted that the enzyme-catalyzed incorporation of amino acids into tRNA was no longer linear with time at 20 minutes after the beginning of the reaction. It was assumed, however, that the kinetics of aminoacylation would be similar for both the pre-incubated and untreated samples of wheat embryo tRNA.

TABLE VI. AMINO ACID ACCEPTOR ACTIVITY OF COMMERCIAL PREPARATIONS OF TRANSFER RNA

Amino Acid	mμmoles amino acid accepted/mg total tRNA*		
	Brewers' Yeast (Boehringer Mannheim)	Bakers' Yeast (General Biochemicals)	E. coli (unstripped) (General Biochemicals)
histidine	---	0.50	---
leucine	---	----	1.2
lysine	1.3	0.75	---
methionine	---	----	0.9
phenylalanine	1.0	----	0.6
serine	1.0	----	---
tyrosine	---	0.62	0.6
valine	1.4	1.00	---

*In the case of the brewers' yeast tRNA, 1 mg of tRNA was assumed to be equivalent to a specific optical density of 25.0 at 260 mμ (d = 1 cm; vol. = 1 ml).

described earlier in the thesis is a highly efficient acceptor of amino acids. Previous studies of wheat embryo tRNA had shown that at least 75% of the chains had adenosine at the 5'-linked terminus, while another 15% had cytidine in this position (Hudson, Gray and Lane, 1965). These end group data indicated that at least 90% of the chains should be capable of accepting amino acids, provided that the crude preparation of wheat embryo aminoacyl-tRNA synthetases also contained the pyrophosphorylase catalyzing the addition of AMP to cytidine-terminated tRNA chains. Since, under optimal conditions, yields of 1 - 2 mμmoles aminoacyl-tRNA/mg added tRNA were observed in the case of certain amino acids, both the amino-acylation studies and the end group analyses support the view that the chemical and physical integrity of wheat embryo tRNA is largely preserved during its isolation. In this regard it is noteworthy that hydrolysis of wheat embryo tRNA by highly-purified snake venom phosphodiesterase has indicated that on the average, over 90% of the chains contain no phosphodiester cleavages (Hudson, Gray and Lane, 1965; Diemer, 1965).

In connection with these studies of the amino acid acceptor activity of wheat embryo transfer RNA, it should be noted that, on the basis of information supplied by Boehringer Mannheim and General Biochemicals, the samples of yeast and E. coli tRNA obtained from these firms also appeared to be active acceptors of amino acids (Table VI).

II. 5-CARBOXYMETHYLURIDINE, A NOVEL NUCLEOSIDE DERIVED FROM YEAST AND WHEAT EMBRYO tRNA

(1) Introduction

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) from internal positions of the ribonucleate chains[1]. 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

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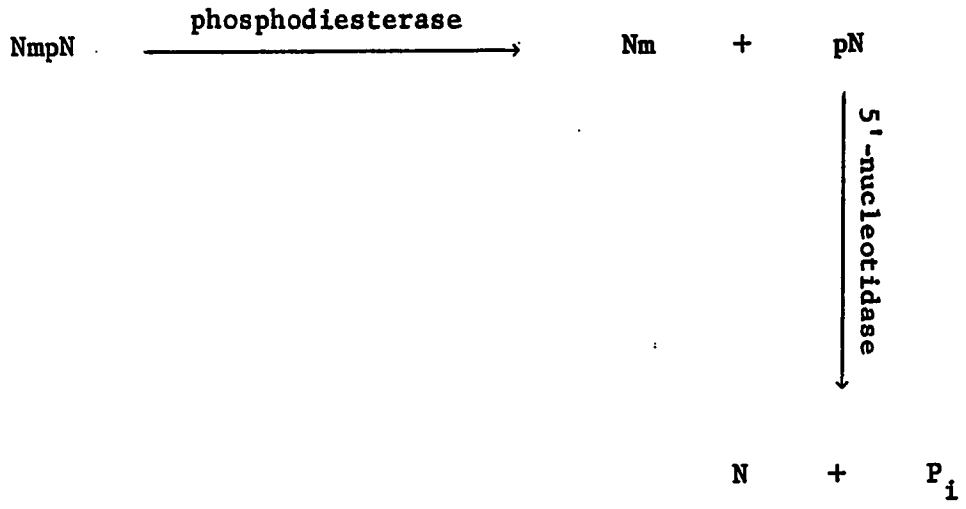
[1]The conditions which we use for alkali hydrolysis (1 M NaOH, 90 hours, room temperature) are sufficient to ensure complete hydrolysis of most of those normal oligonucleotides (e.g., ApAp) which are only slowly hydrolyzed by alkali (Lane and Butler, 1959). However, it is relevant to point out that 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. $10^{-3}hr^{-1}$).

charges at pH 7.8 (N, 0; Np, -2; NmpNp, -3; 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, Gray and Lane, 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, Gray and Lane, 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 (Scheme 1).

SCHEME 1



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.

It was found, in fact, that T? is <u>not</u> a dinucleoside phosphate, but instead a carboxymethyl-containing nucleoside (see "Note Added in Proof", Gray and Lane, 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.

Part II of the thesis 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.

(2) Materials

Brewers' yeast "soluble" RNA was purchased from Boehringer Mannheim Corporation. Wheat embryo "soluble" RNA was prepared according to the procedure outlined in Part I. *E. coli* "soluble" RNA 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. For this reason, 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, by vol.), 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).

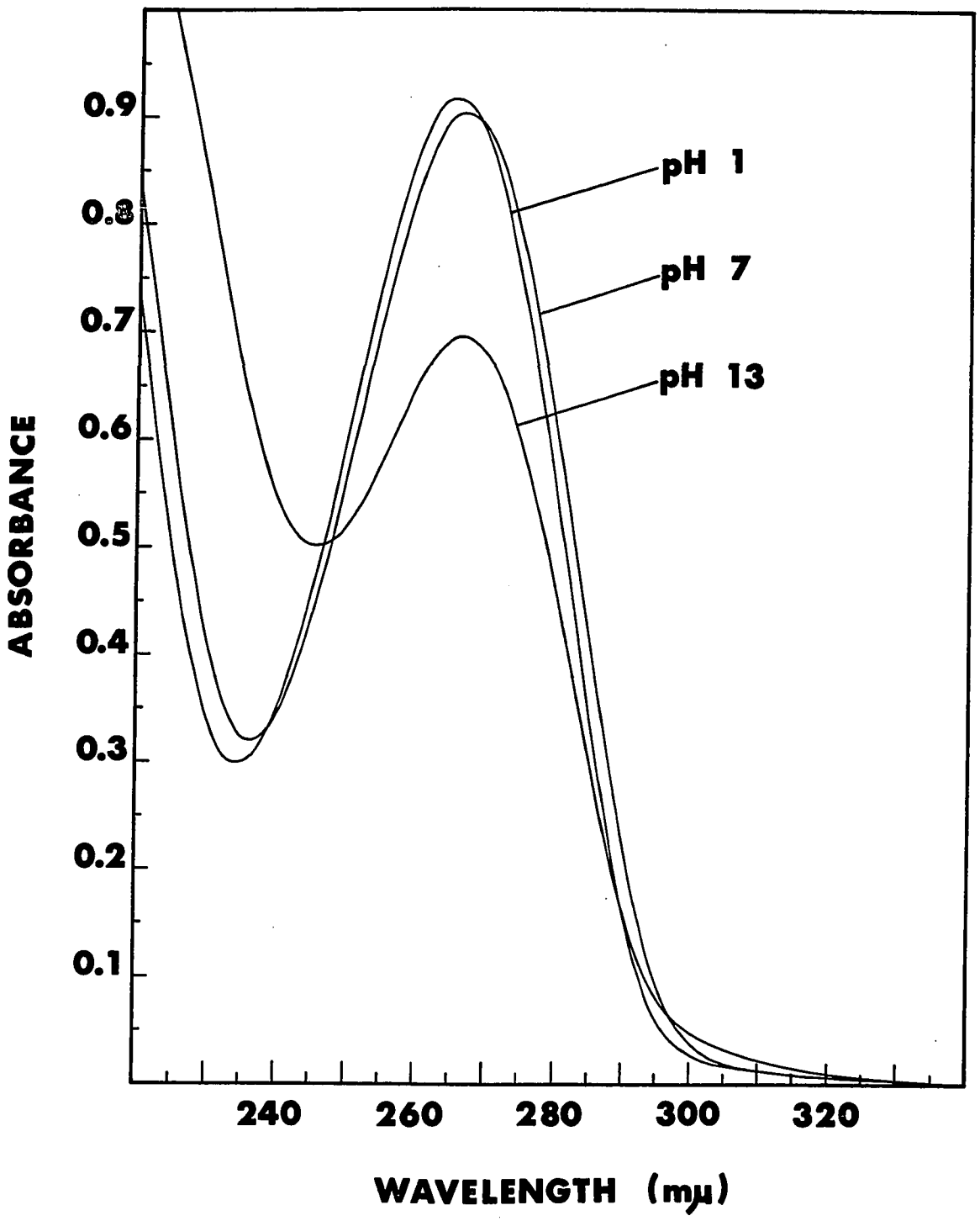
Thiourea, diethyl succinate, and ethyl formate were purchased from Aldrich Chemical Co., Inc. Pyridine (Spectro-quality Reagent) was obtained from Matheson, Coleman and Bell.

(3) 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 nmr spectra reported are proton spectra and chemical shifts are relative to tetramethylsilane as external standard at $\tau = 10$. Infrared spectra were determined on a Perkin-Elmer model 421 spectrophotometer. 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 the major peaks. 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 volts for 1 - 2 hours. The systems used for paper chromatography have previously been described in detail (Gray and Lane, 1967). The developing solvents were 95% ethyl

FIGURE 3. Ultraviolet absorption spectra of 5-carboxymethyluridine ("T?") isolated from brewers' yeast tRNA



λ_{max} (mμ) = 265 (pH 1), 266.5 (pH 7), 266.5 (pH 13)

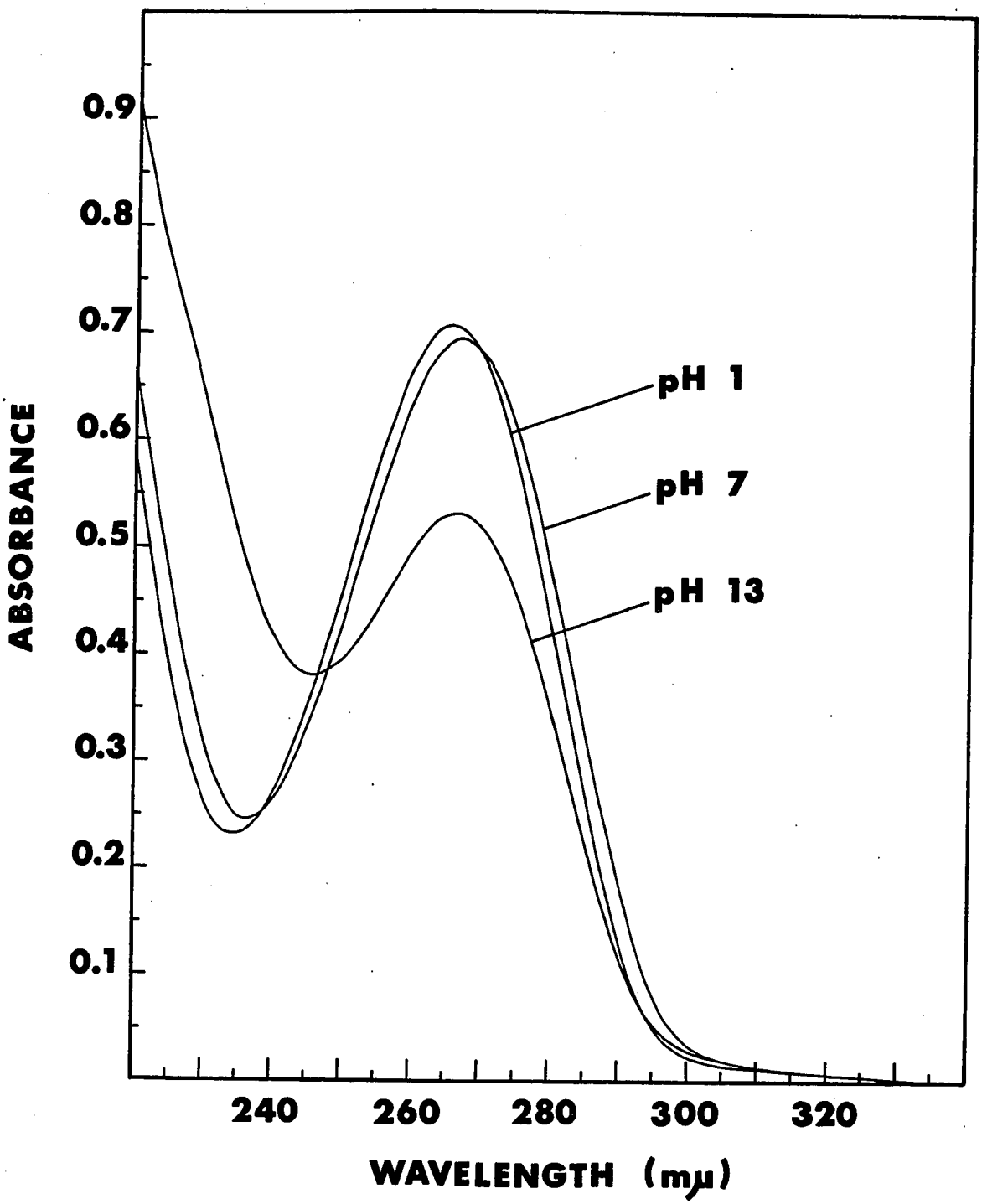
λ_{min} (mμ) = 234 (pH 1), 236 (pH 7), 245.5 (pH 13)

alcohol : water, 4:1, by vol. (System 1) and saturated ammonium sulphate : 2-propanol, 40:1, by vol. (System 2); they were used in conjunction with Whatman No. 1 chromatography paper which had been impregnated with ammonium sulphate (Lane, 1963).

(4) General Approach to the Characterization of 5-Carboxymethyluridylate, 5-Carboxymethyluridine, and 5-Carboxymethyluracil Derived from tRNA

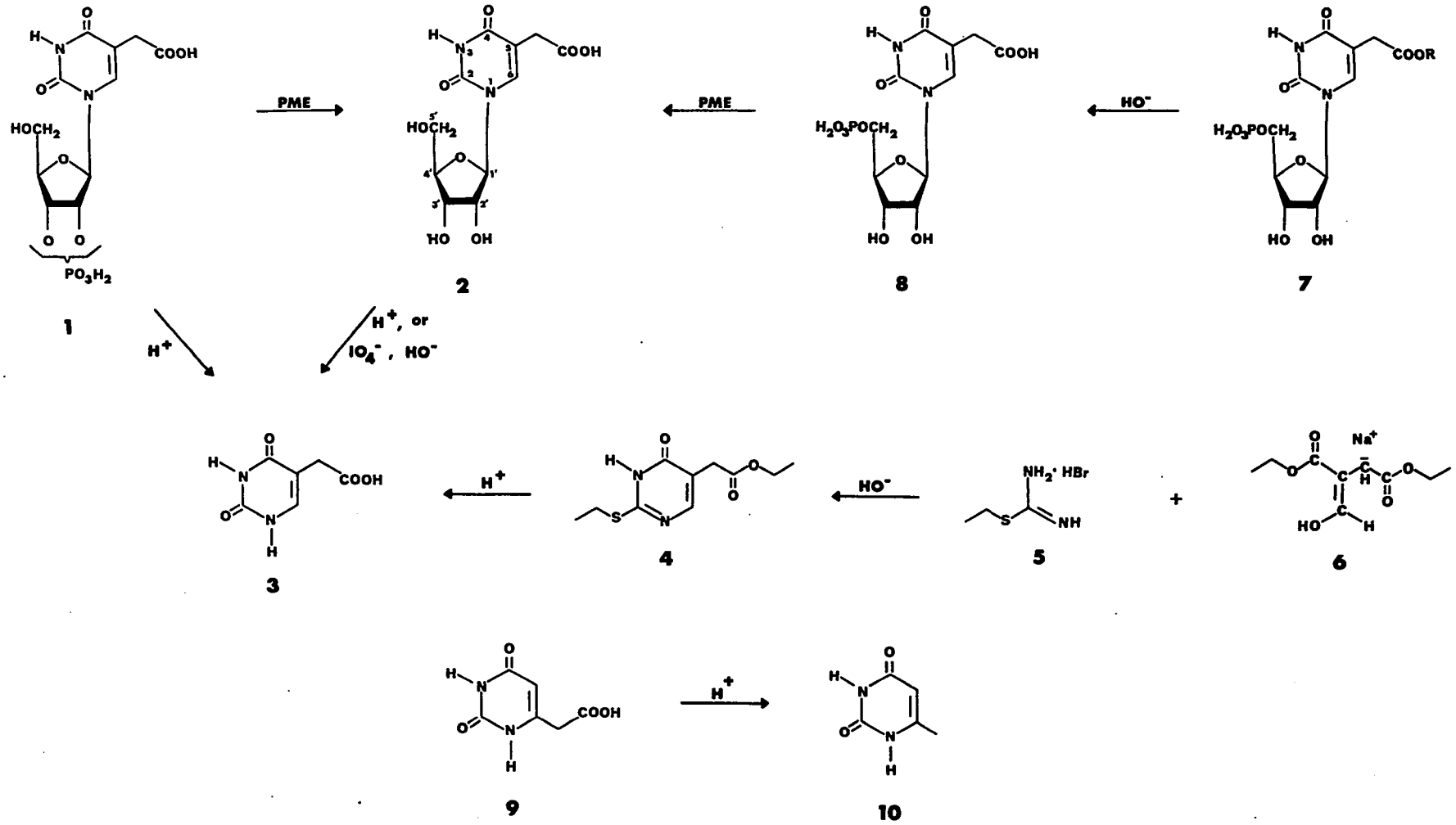
The unknown compound (T?) mentioned in the introduction to this part of the thesis was very similar to ribothymidine in its ultraviolet spectral properties (Figures 3 and 4), and was isolated from a fraction that was initially believed to be composed exclusively of dinucleoside phosphates. Since, as with dinucleoside phosphates, T? had a single negative charge at pH 7, it was initially thought 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, Brownlee and Barrell, 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?

FIGURE 4. Ultraviolet absorption spectra of 5-carboxy-methyluridine ("T?") isolated from wheat embryo tRNA



might be a nucleoside bearing a negative charge at pH 7. If T? were a nucleoside, it would of course be unaffected by non-specific 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 pH 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

FIGURE 5



LEGEND OF FIGURE 5

A diagram illustrating the synthesis of 5-carboxymethyluracil, and the structures and interrelations of the various carboxymethyl uracil derivatives. The compounds are:

1, 5-carboxymethyluridine 2'(3')-phosphate (cm^5Up)

2, 5-carboxymethyluridine (cm^5U)

3, 5-carboxymethyluracil (cm^5u)

4, ethyl 2-ethylthio-4-oxypyrimidine-5-acetate

5, S-ethylthiourea hydrobromide

6, diethyl formylsuccinate, sodium salt

7, unspecified carboxylate ester of 5-carboxymethyluridine 5'-phosphate

8, 5-carboxymethyluridine 5'-phosphate (pcm^5U)

9, 6-carboxymethyluracil (cm^6u)

10, 6-methyluracil (m^6u)

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 the 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 are discussed

SCHEME 2

YEAST tRNA

↓ HO^-

ALKALI HYDROLYSATE
(22,550 A_{260} units)

↓

FRACTIONATION ON DEAE-CELLULOSE ACCORDING TO NET CHARGE AT pH 7.8

Eluent (1)	Eluent (2)	Eluent (3)	Eluent (4)
↓	↓	↓	↓
Fraction 1	Fraction 2	Fraction 3	Fraction 4
(net charge = 0)	(net charge = -2)	(net charge = -3)	(net charge = -4)
417 A_{260} units (1.85 %)	21,360 A_{260} units (94.64 %)	427 A_{260} units (1.89 %)	365 A_{260} units (1.62 %)
nucleosides (N); trace quantity of bases (n)	nucleoside 2'(3')-monophosphates (Np)	alkali-stable dinucleotides (NmpNp); cm^5Up	nucleoside 2'(3'),5'-bisphosphates (pNp)

Desalted on DEAE-formate

↓

FRACTIONATION ON DEAE-FORMATE ACCORDING TO NET CHARGE AT pH 1.8

Eluent (5) → Subfraction 1 (net charge = 0) + Subfraction 2 (net charge = -0.5) + Subfraction 3 (net charge = -1)

Eluent (6) → Subfraction 4

Subfraction 1	Subfraction 2	Subfraction 3	Subfraction 4
net charge = 0	net charge = -0.5	net charge = -1	
72 A_{260} units	69 A_{260} units	128 A_{260} units	142 A_{260} units
CmpCp	CmpGp	CmpUp	GmpGp
CmpAp	GmpAp	AmpUp	UmpGp
AmpAp		UmpAp	UmpUp
		cm^5Up	

Subfraction 3 → Eluent removed in vacuo → PAPER CHROMATOGRAPHY, SYSTEM B

Band 1 : AmpUp
Band 2 : UmpAp
Band 3 : CmpUp
Band 4 : cm^5Up

Band 4 → Eluted with water, desalted on DEAE-carbonate → 48 A_{260} units

---

Eluents: (1) 0.025 M Tris formate (pH 7.8); (2) 0.085 M Tris formate (pH 7.8), 7.3 M in urea; (3) 0.17 M Tris formate (pH 7.8), 6.6 M in urea; (4) 0.35 M Tris formate (pH 7.8), 5.2 M in urea; (5) 1 M formic acid (pH 1.8); (6) 1 M pyridinium formate (pH 4.5), after washing column with water

LEGEND OF SCHEME 2

Brewers' yeast tRNA (1.07 g, purified by chromatography on DEAE-cellulose) was dissolved in water (25 ml) and 10 M sodium hydroxide (2.8 ml) was added. The alkaline solution was allowed to stand at room temperature for 90 hours, after which time the resulting alkali hydrolysate was adjusted to pH 7.8 with conc. formic acid, diluted with water to reduce the formate ion concentration to 0.025 M, and applied to a 4.25 cm (i.d.) X 20 cm (h.) column of DEAE-formate, equilibrated with 0.025 M Tris formate (pH 7.8). Fraction 1 was not retained by the column, while Fractions 2 - 4 were eluted in a stepwise manner using urea-containing formate buffers of increasing ionic strength. After diluting Fraction 3 with a three-fold excess of water, the dinucleotides (plus cm^5Up) were re-adsorbed to a 4.25 cm (i.d.) X 10 cm (h.) column of DEAE-formate. The column was washed with water to remove Tris formate and urea, and the salt-free dinucleotides (plus cm^5Up) were then eluted with 1 M pyridinium formate (pH 4.5), a volatile buffer easily removable *in vacuo*. Further resolution of the components of Fraction 3 at pH 1.8 is described in Figure 6.

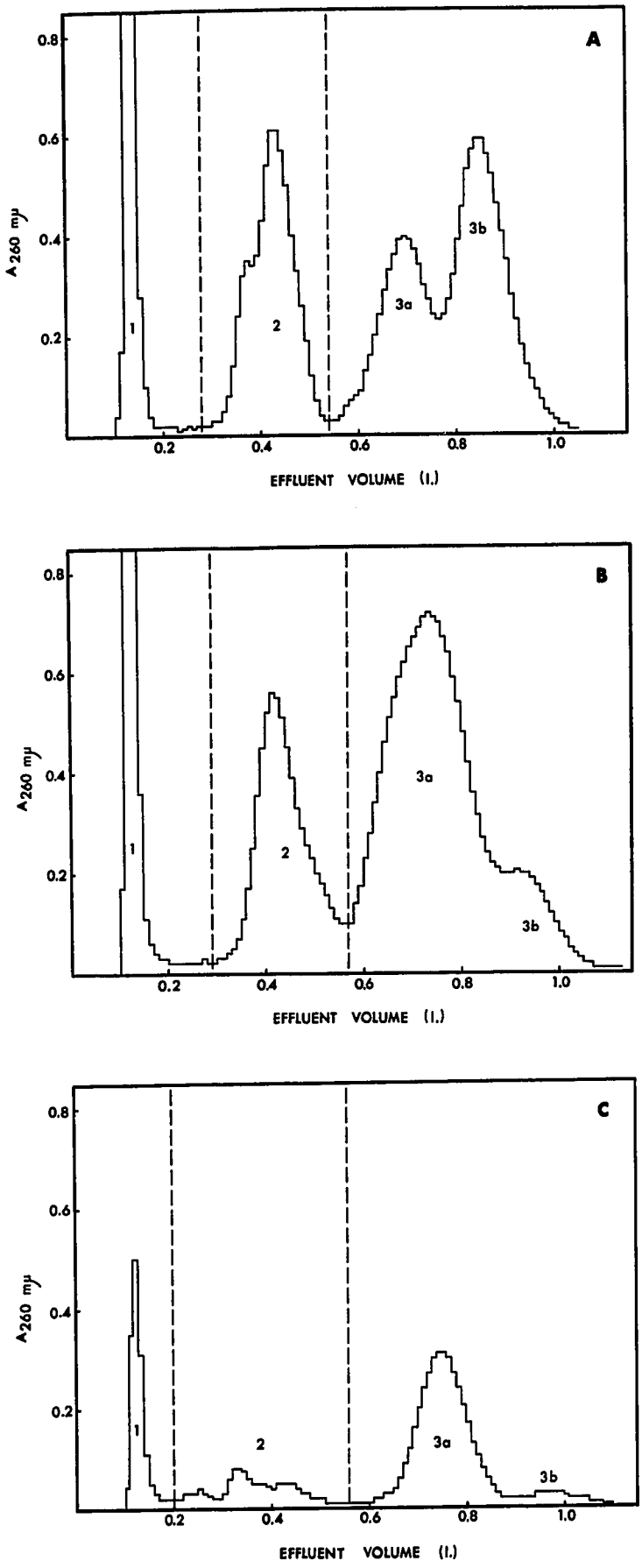
in some detail toward the end of this part of the thesis, and a description of the difficulties encountered in attempting to isolate 5-carboxymethyluridine 5'-phosphate from a venom phosphodiesterase hydrolysate of tRNA is also presented.

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

(5) Isolation of 5-Carboxymethyluridine 2'(3')-phosphate from Alkali Hydrolysates of tRNA

Before alkali hydrolysis, all tRNA preparations were chromatographed on DEAE-cellulose as described in Part I (3), in order to remove any low molecular weight nucleotides and/or contaminant rRNA (Glitz and Dekker, 1963; Bell, Tomlinson and Tener, 1964). Chemical and physicochemical characterizations of the purified preparations of wheat embryo tRNA and brewers' yeast tRNA have been reported (Hudson, Gray and Lane, 1965; Kay and Oikawa, 1966; Gray and Lane, 1967). As indicated by the data in Part I (5), the tRNA preparations from wheat embryo and yeast had similar amino acid accepting capacities when assayed with their homologous amino acid activating enzymes. 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

FIGURE 6



LEGEND OF FIGURE 6

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 gram 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 on 2.0 cm (i.d.) X 30 cm (h.) columns of DEAE-formate, and elution was begun with 1 M formic acid (pH 1.8), at a flow rate of 4 ml/min. Ten ml fractions were collected every 2.5 minutes. 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-carboxymethyl-uridylate 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).

dinucleotides by chromatography on DEAE-cellulose at pH 1.8, and (iv) recovery of salt-free compounds from column eluates. These procedures, as applied to yeast tRNA, are summarized in Scheme 2.

Figure 6 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 behaviour 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 6, A) 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 (Figure 7). The nucleotide was eluted from chromatograms with

FIGURE 7

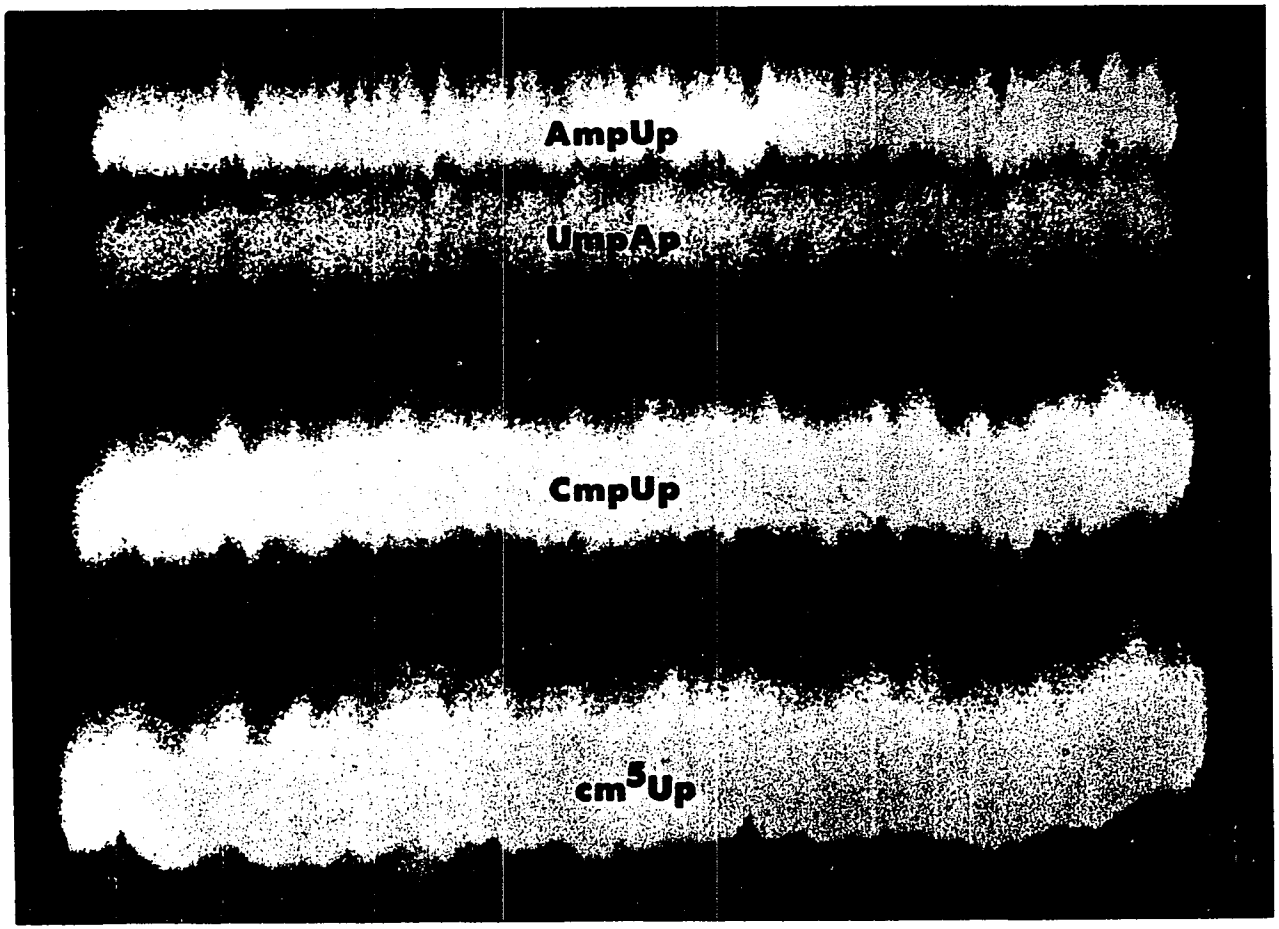
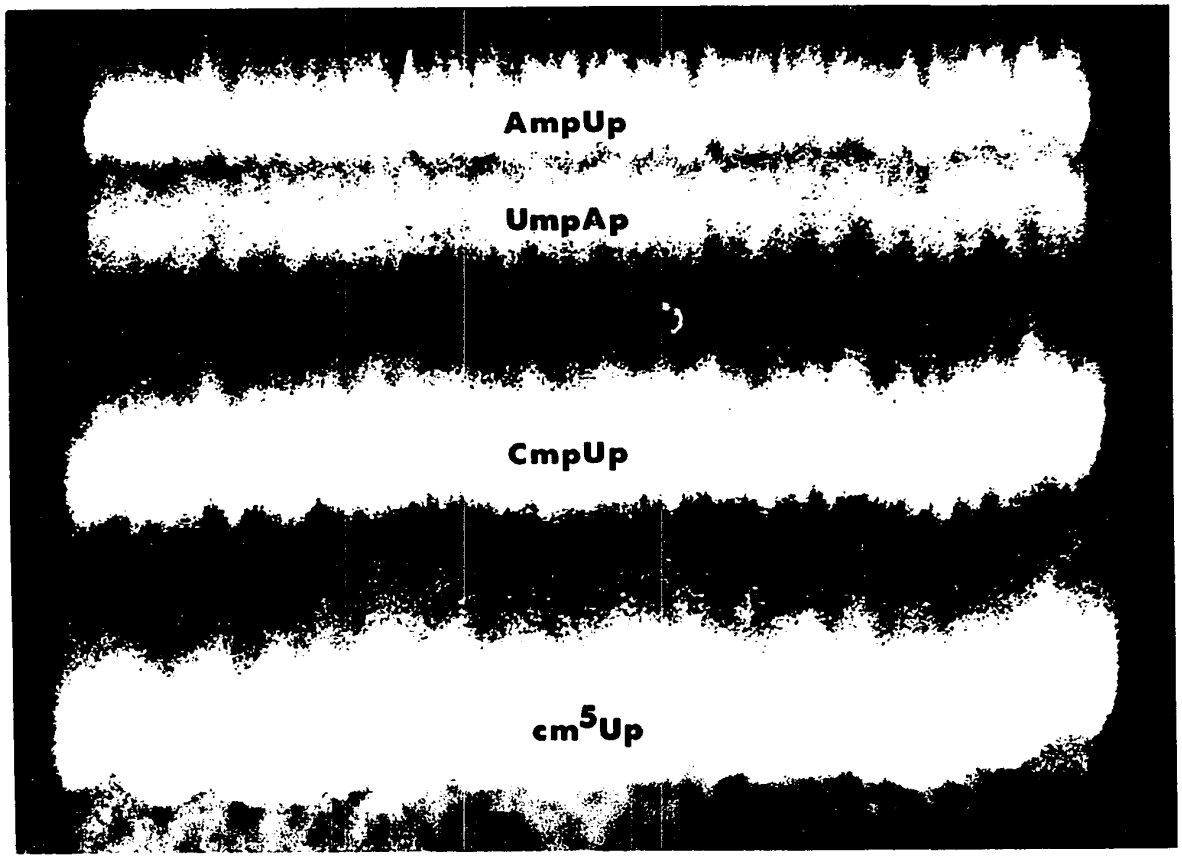


FIGURE 7



LEGEND OF FIGURE 7

An ultraviolet contact photograph illustrating the paper chromatographic separation of 5-carboxymethyluridine 2'(3')-phosphate (5-carboxymethyluridylate) from the alkali-stable dinucleotides (AmpUp, UmpAp, CmpUp) present in Subfraction 3 after DEAE-cellulose chromatography (pH 1.8) of the alkali-stable dinucleotides isolated from brewers' yeast tRNA (see Scheme 2 and Figure 6, A). A solution containing approximately 200 A_{260} units of Subfraction 3 was streaked on a large sheet (ca. 18 inches wide) of ammonium sulphate-impregnated Whatman No. 1 chromatography paper, and the chromatogram was developed in System 2 (descending chromatography). The origin (not shown in the photograph) was above AmpUp, the slowest-moving of the components of Subfraction 3 during paper chromatography in System 2.

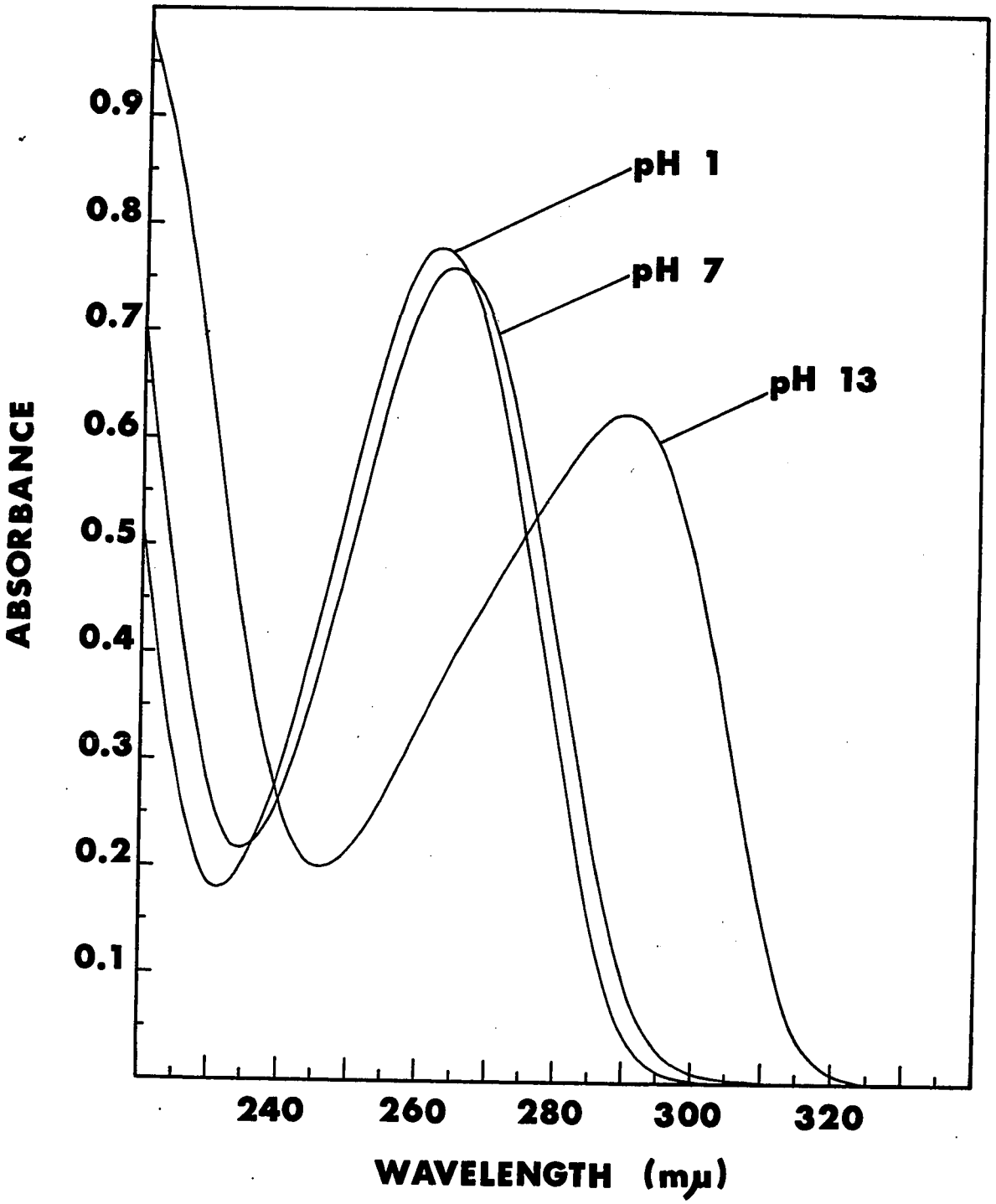
LEGEND OF FIGURE 7

An ultraviolet contact photograph illustrating the paper chromatographic separation of 5-carboxymethyluridine 2'(3')-phosphate (5-carboxymethyluridylate) from the alkali-stable dinucleotides (AmpUp, UmpAp, CmpUp) present in Subfraction 3 after DEAE-cellulose chromatography (pH 1.8) of the alkali-stable dinucleotides isolated from brewers' yeast tRNA (see Scheme 2 and Figure 6, A). A solution containing approximately 200 A_{260} units of Subfraction 3 was streaked on a large sheet (ca. 18 inches wide) of ammonium sulphate-impregnated Whatman No. 1 chromatography paper, and the chromatogram was developed in System 2 (descending chromatography). The origin (not shown in the photograph) was above AmpUp, the slowest-moving of the components of Subfraction 3 during paper chromatography in System 2.

water, and the resulting aqueous eluate was freed of ammonium sulphate (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 sulphate 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. It has been 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 bisphosphate 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 bisphosphate is

FIGURE 8



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

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.

(6) 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 and wheat embryo tRNA are shown in Figures 3 and 4, respectively.

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 hours at 100°, in a sealed, evacuated tube (Furukawa et al, 1966). The ultraviolet absorption spectra of 5-carboxymethyluracil isolated from wheat embryo tRNA are shown in Figure 8.

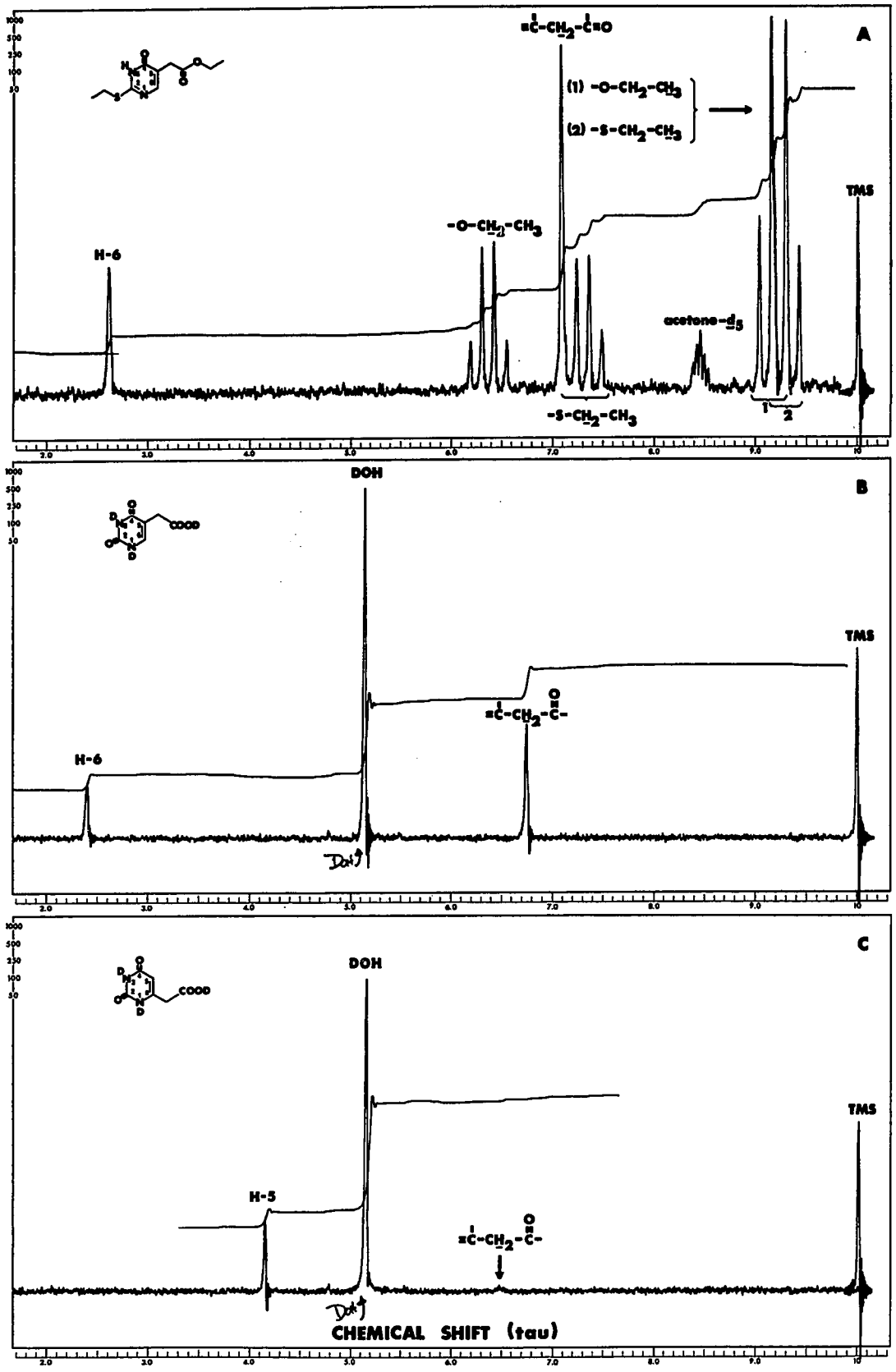
(7) Synthesis and Characterization of 5-Carboxymethyluracil

5-Carboxymethyluracil was prepared as described by Johnson and Speh (1907). The synthesis is outlined in Figure 5 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 to 5-carboxymethyluracil (3) by refluxing with hydrochloric acid.

S-Ethylthiourea hydrobromide was synthesized by alkylation of thiourea with ethyl bromide in absolute ethanol (Brand and Brand, 1955). After removal in vacuo of excess ethyl bromide, ethanol, and any ethyl thiocyanate, the residual oil was allowed to crystallize at room temperature, and after air drying, the solid was used without further purification. The sodium salt of diethyl formylsuccinate was prepared by slow addition of a mixture of 167.5 ml (174 g, 1 mole) diethyl succinate and 80 ml (74 g, 1 mole) ethyl formate to a suspension of 23 g (1 mole) metallic sodium in 500 ml anhydrous ethyl ether. Evolution of hydrogen ensued and the reaction mixture turned a deep red. After allowing the mixture to stand overnight at room temperature, a small amount of residual sodium was destroyed by addition of 95% ethanol, and the sodium salt of diethyl formylsuccinate was extracted into cold water.

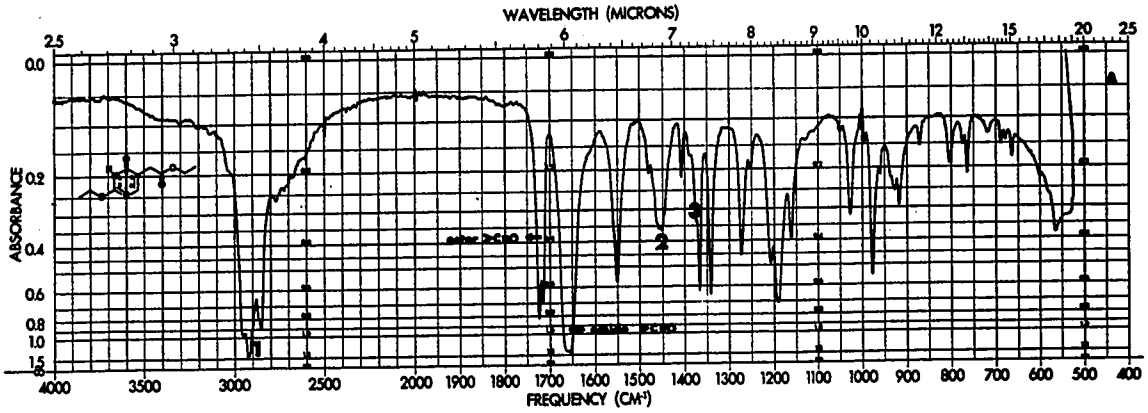
Solid S-ethylthiourea hydrobromide (200 g, ca. 1 mole) was added to an aqueous solution of the sodium salt of diethyl

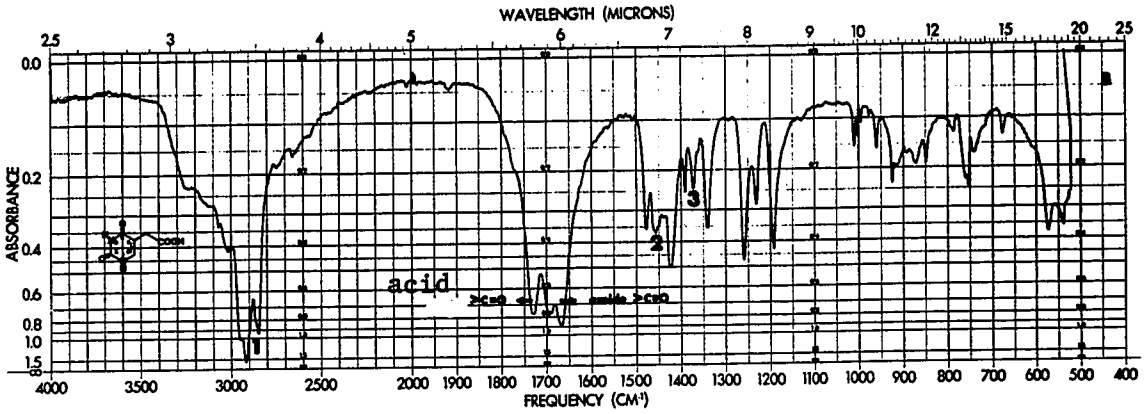
FIGURE 9. Nuclear magnetic resonance (nmr) spectra of ethyl 2-ethylthio-4-oxypyrimidine-5-acetate in acetone-$\underline{d}_6$ (A), 5-carboxymethyluracil in D_2O (B), and 6-carboxymethyluracil in D_2O (C)

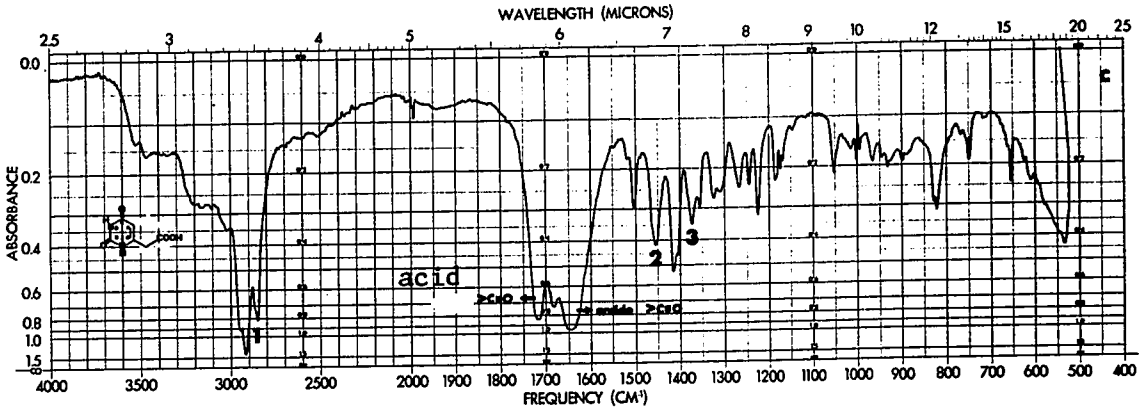


formylsuccinate (240 g, assuming complete reaction, in ca. 750 ml water), prepared as described above. To the resulting solution, cold aqueous potassium hydroxide (100 ml containing 1 mole) was added. The deeply-colored reaction mixture was allowed to stand at room temperature for 1 - 3 days, after which time the mixture was heated on a steam-bath for 3 hours. Upon cooling the solution to 4°, crystals of ethyl 2-ethylthio-4-oxypyrimidine-5-acetate deposited, and these were collected by filtration and then washed with cold water. When the weakly alkaline filtrate was acidified with acetic acid, more of the thiopyrimidine separated. This second crop of crystals was redissolved by brief heating, the resulting solution was filtered to remove some insoluble material, and the thiopyrimidine was allowed to recrystallize in the cold. Combination of the two crops of crystals gave ca. 80 g of crude pyrimidine (ca. 30% of theoretical, based on diethyl succinate), which formed a clear, reddish solution when dissolved in hot ethanol. Colored impurities were removed by treatment of the solution with Norit-A charcoal, and after filtration, a clear, colorless solution was obtained from which ethyl 2-ethylthio-4-oxypyrimidine-5-acetate crystallized in the form of slender, colorless needles. The compound was finally recrystallized several times from ethanol, 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.

FIGURE 10. Infrared spectra (Nujol mulls) of ethyl 2-ethylthio-4-oxypyrimidine-5-acetate (A), 5-carboxymethyluracil (B), and 6-carboxymethyluracil (C). The numbers on each spectrum refer to absorptions due to the mulling agent, Nujol [C-H stretching (1); C-H bending (2 and 3)].







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)

Nuclear magnetic resonance spectrum (acetone-$\underline{d}_6$) (Figure 9, A): τ 2.60 (1H, singlet, H-6), 6.37 (2H, quartet, -O-C$\underline{H}_2$-CH_3, $\underline{J}$ = 7 cps), 7.08 (2H, singlet, =C-C$\underline{H}_2$-C(=O)-), 7.29 (2H, quartet, -S-C$\underline{H}_2$-CH_3, $\underline{J}$ = 7 cps), 9.17 and 9.30 (6H, -O-CH_2-C$\underline{H}_3$ and -S-CH_2-C$\underline{H}_3$, two overlapping triplets, $\underline{J}$ = 7 cps)

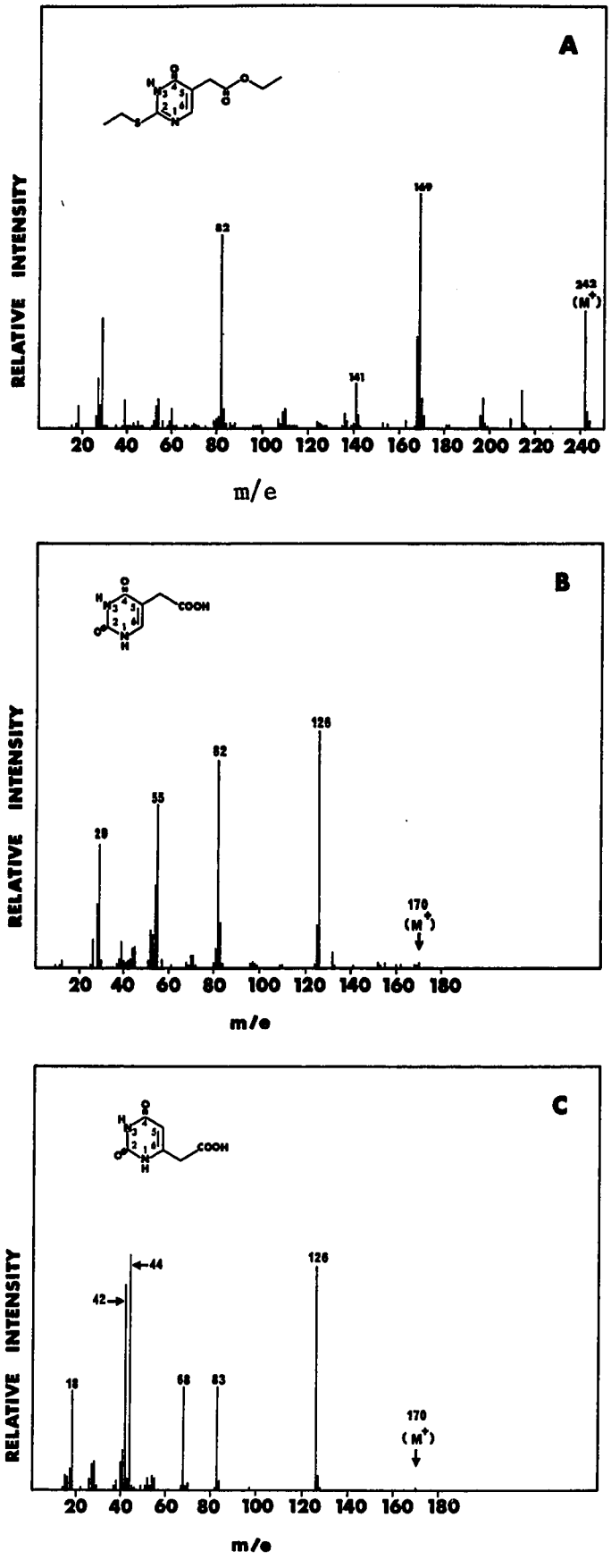
Infrared spectrum (Figure 10, A): λ_{max}^{Nujol} (μ) = 5.81 and 5.83 (carbonyl stretching; -CH_2-C(=O)-O-CH_2-), 5.99 - 6.05 (carbonyl stretching; -C(=O)-NH-), 6.44, 7.32, 7.45, 7.86, 8.28, 8.40, 8.62, 9.73, 10.25

Mass spectrum (direct probe, source temperature 175°) (Figure 11, A): $\underline{m/e}$ 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), 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 conc. hydrochloric acid. The product precipitated as a white powder after $\underline{ca}$. 3 hours of heating. After cooling the reaction mixture at 4° for several hours, the precipitate was collected by filtration and washed with cold water. The crude acid was dissolved in boiling water to form a clear, slightly yellow solution and charcoal was

FIGURE 11

Mass spectra of ethyl 2-ethylthio-4-oxypyrimidine-5-acetate (A), 5-carboxymethyluracil (B), and 6-carboxymethyluracil (C)



added to remove the colored impurities. After filtration, 5-carboxymethyluracil separated from the clear, colorless filtrate in the form of white, granular crystals, mp 311.0 - 311.5°, with decomp. (lit. (Johnson and Speh, 1907) mp 315 - 320°, with decomp.). 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)

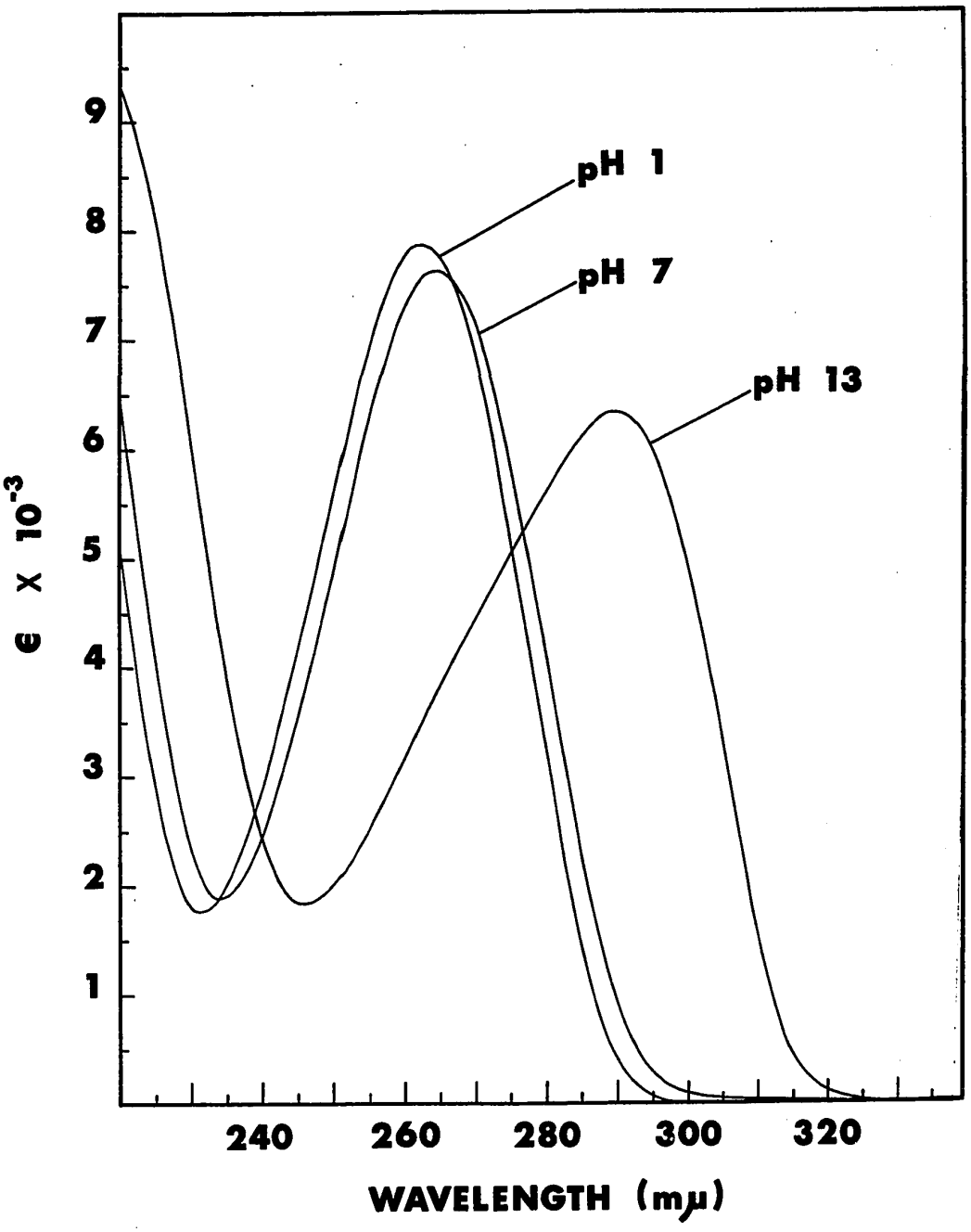
Nuclear magnetic resonance spectrum (D_2O) (Figure 9, B): τ 2.40 (1H, singlet, H-6), 6.75 (2H, singlet, $=\overset{|}{C}-\underline{CH_2}-\overset{O}{\overset{\|}{C}}-$)

Infrared spectrum (Figure 10, B): λ_{max}^{Nujol} (μ) = 5.78 (carbonyl stretching; $-CH_2-\overset{O}{\overset{\|}{C}}-OH$), 5.91 and 6.00 (carbonyl stretching; $-\overset{O}{\overset{\|}{C}}-NH-$), 6.77, 7.03, 7.95, 8.39, 13.3

Mass spectrum (direct probe, source temperature 200°) (Figure 11, B): m/e 170 (M^+; 2), 126 (loss of 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), 18 (14)

Ultraviolet spectrum (Figure 12): λ_{max} (mμ) = 262 (pH 1), 264 (pH 7), 290 (pH 13); λ_{min} (mμ) = 231 (pH 1), 234 (pH 7), 246 (pH 13); ϵ_{260} = 7240 in 0.01 M phosphate buffer (pH 7.45)

FIGURE 12



Ultraviolet absorption spectra of synthetic 5-carboxymethyluracil

(8) Characterization of 6-Carboxymethyluracil and Comparison with Synthetic 5-Carboxymethyluracil

Commercially-available 6-carboxymethyluracil was freed from traces of 6-methyluracil as described in Part II (2). The purified product was then characterized in order to distinguish it from the synthetic 5-carboxymethyluracil described above.

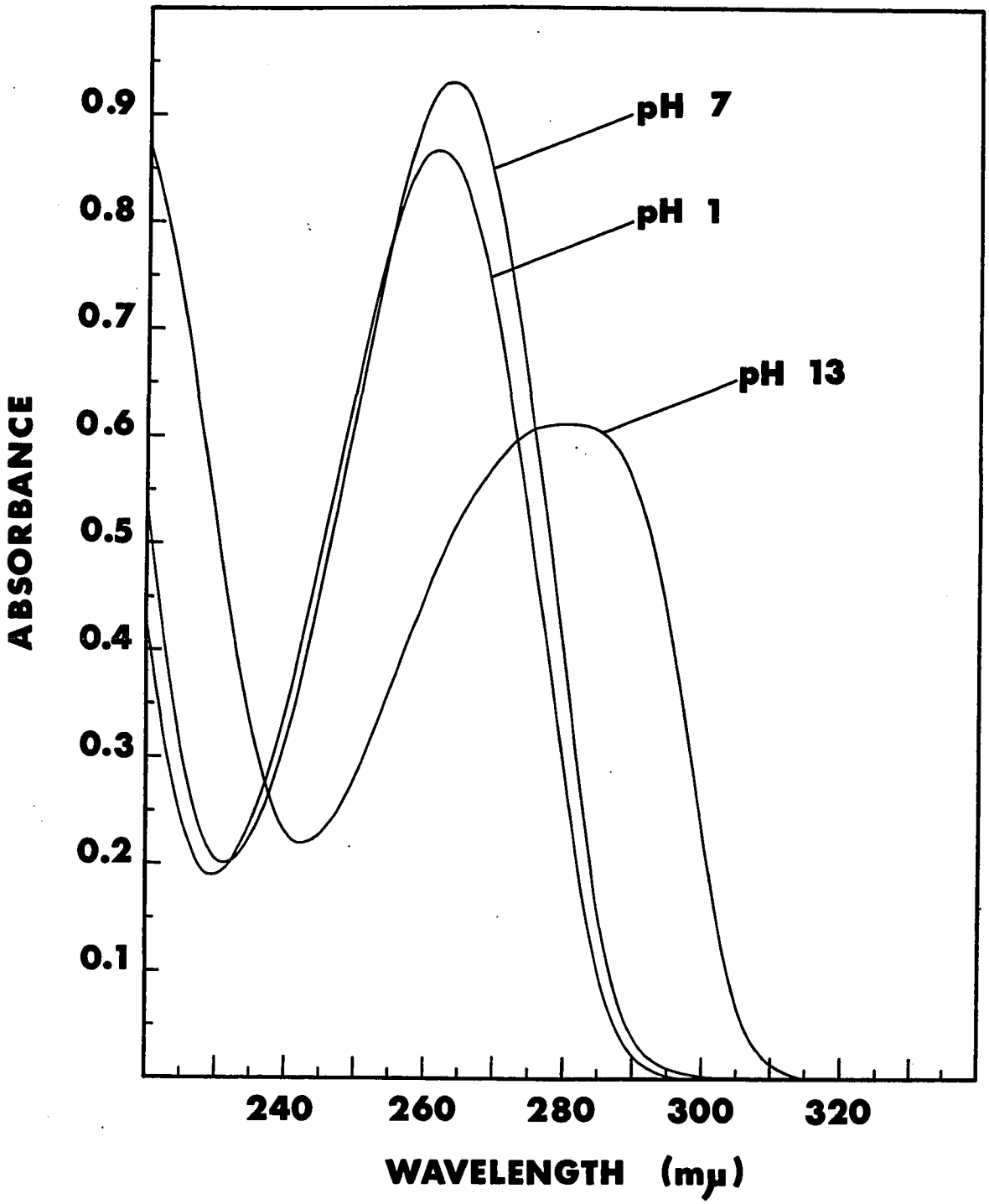
Anal. Calcd. for $C_6H_6N_2O_4$: mol. wt. 170.0328. Found: mol. wt. 170.0328 (mass spectrometry)

Nuclear magnetic resonance spectrum (D_2O) (Figure 9, C): τ 4.13 (1H, 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.

Infrared spectrum (Figure 10, C): λ_{max}^{Nujol} (μ) = 5.82 (carbonyl stretching; $-CH_2-\overset{O}{\overset{\|}{C}}-OH$), 5.94 and 6.04 - 6.09 (carbonyl stretching; $-\overset{O}{\overset{\|}{C}}-NH-$), 6.64, 7.07, 8.18, 12.2

Mass spectrum (direct probe, source temperature 200°)

FIGURE 13



Ultraviolet absorption spectra of 6-carboxymethyluracil

(Figure 11, C): 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), 18 (42)

Ultraviolet spectrum (Figure 13): λ_{max} (mμ) = 261 (pH 1), 263 (pH 7), 276.5 - 283 (pH 13); λ_{min} (mμ) = 229 (pH 1), 231 (pH 7), 242 (pH 13)

5-Carboxymethyluracil (3) and 6-carboxymethyluracil (9) can be readily distinguished from one another on the basis of their nmr, ir, and uv spectra. Of particular note are the nmr spectra (Figure 9), 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 τ = 2.40, respectively (Bhacca et al, 1963), and since the nmr 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 (Figure 10) 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 presence of this carboxylic absorption becomes more apparent when the infrared spectra of these two compounds are compared with the infrared spectrum of ethyl 2-ethylthio-4-oxypyrimidine-5-acetate, which has no carboxylic O-H stretching absorption in the 3.0 - 3.5 μ region.

The ultraviolet spectra of 5-carboxymethyluracil (Figure 12) and 6-carboxymethyluracil (Figure 13) 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-carboxymethyluracil. 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 (Figure 11) 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.

(9) Comparison of 5- and 6-Carboxymethyluracil with the Base Derived from tRNA

TABLE VII. RELATIVE ELECTROPHORETIC MOBILITIES OF VARIOUS URACIL DERIVATIVES

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^5Up (yeast tRNA)	+0.45	+0.63	+1.30	+1.65	+1.58
cm^5Up (wheat embryo tRNA)	+0.49	+0.67	+1.36	+1.70	+1.63
m^5Up	+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^5U (yeast tRNA)	-0.08	+0.25	+0.51	+0.53	+1.02
cm^5U (wheat embryo tRNA)	-0.05	+0.22	+0.52	+0.52	+1.04
m^5U	-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^5u (yeast tRNA)	-0.03	+0.31	+0.86	+1.13	+0.92
cm^5u (wheat embryo tRNA)	-0.04	+0.31	+0.87	+1.14	+0.98
cm^5u (synthetic)	-0.05	+0.31	+0.80	+1.10	+0.92
cm^6u	-0.03	+0.75	+0.96	+1.19	+1.13
m^5u	-0.04	-0.02	-0.15	-0.11	0.00
m^6u	-0.04	-0.10	-0.15	-0.07	+0.08

The picrate marker was assigned a mobility of +1.00 at each pH value. Positive values indicate that migration was towards the anode, and negative values indicate migration towards 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); (E) 0.025 M sodium (tetra)borate (pH 9.2). Compounds (ca. 0.05 μmole, in 25 μl) were spotted on 3.0 cm X 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^5U" is 5-carboxymethyluridine.

The base isolated by acid hydrolysis of the presumed 5-carboxymethyluridylate (from either yeast or wheat embryo tRNA) had ultraviolet spectra (Figure 8) virtually identical with those of synthetic 5-carboxymethyluracil (Figure 12) at pH values of 1, 7, and 13, and quite different from those of 6-carboxymethyluracil (Figure 13) 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 behaviour (Tables VII and VIII; see also Figures 16 and 19). 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 (Figure 16), indicating that the 6-isomer is a substantially stronger acid than the 5-isomer.

The natural base also paralleled 5-carboxymethyluracil in its behaviour toward treatment with acid. Synthetic 5-carboxymethyluracil was stable under the conditions of acid hydrolysis used for the production of the natural base from 5-carboxymethyluridylate or 5-carboxymethyluridine (6 N HCl, 100°, 6 hours), since more than 95% of the compound could be

FIGURE 14

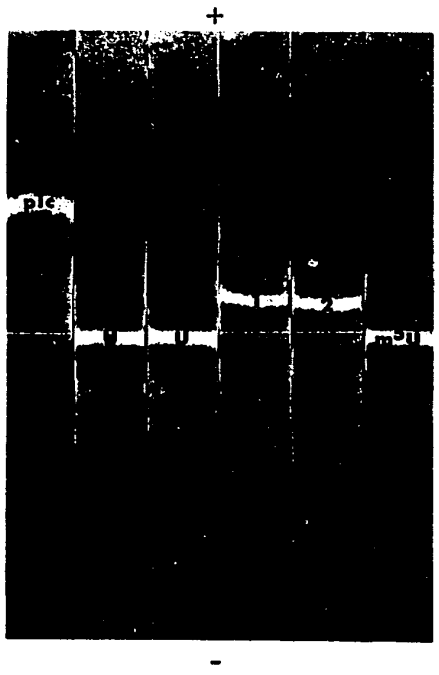


FIGURE 15

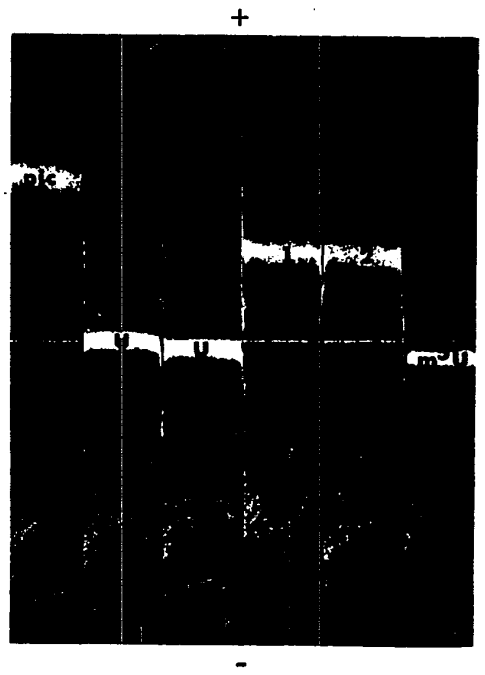


FIGURE 16

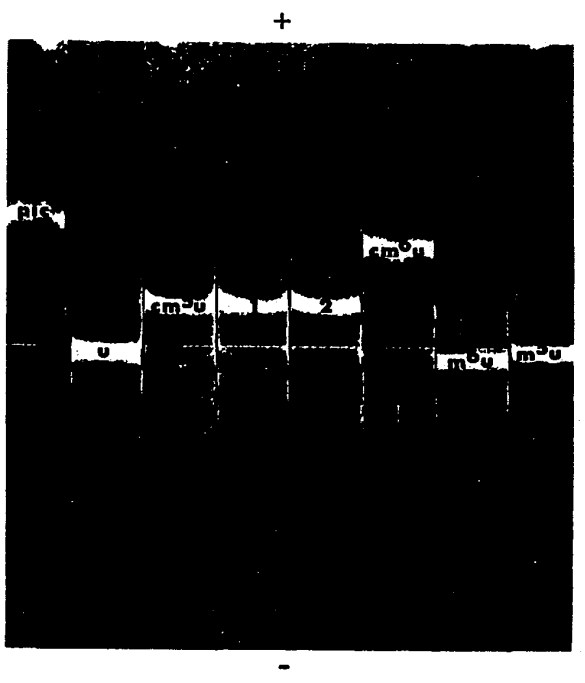


FIGURE 14

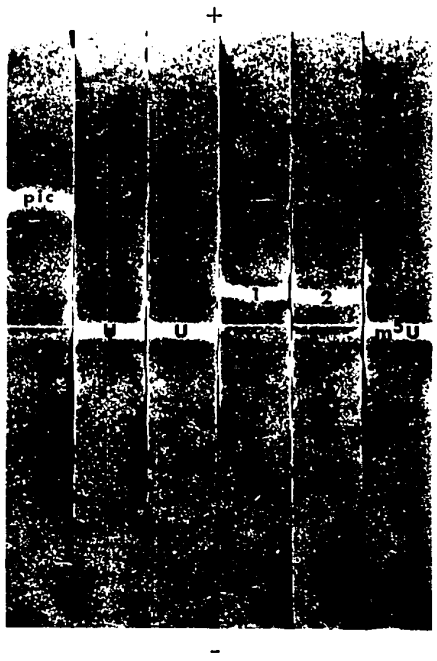
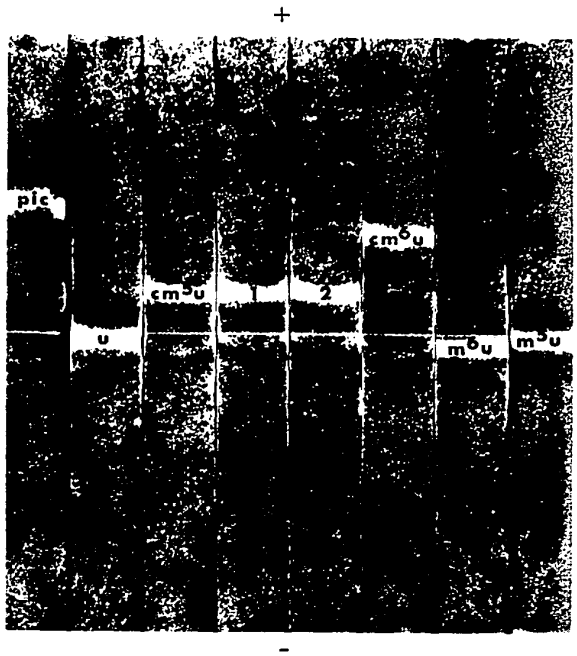


FIGURE 15

FIGURE 16



LEGEND OF FIGURE 14

An ultraviolet contact photograph depicting the paper electrophoretic mobilities of various nucleosides in 0.025 M ammonium formate buffer (pH 3.5). From left to right, the compounds are: picrate marker (pic), pseudouridine (ψ), uridine (U), 5-carboxymethyluridine from yeast tRNA (1), 5-carboxymethyluridine from wheat embryo tRNA (2), and 5-methyluridine (m^5U).

LEGEND OF FIGURE 15

An ultraviolet contact photograph depicting the paper electrophoretic mobilities of various nucleosides in 0.025 M ammonium formate buffer (pH 9.2). The compounds are the same as those in Figure 14 (see above).

LEGEND OF FIGURE 16

An ultraviolet contact photograph depicting the paper electrophoretic mobilities of various pyrimidine bases in 0.025 M ammonium formate buffer (pH 3.5). From left to right, the compounds are: picrate marker (pic), uracil (u), synthetic 5-carboxymethyluracil (cm^5u), 5-carboxymethyluracil from yeast tRNA (1), 5-carboxymethyluracil from wheat embryo tRNA (2), 6-carboxymethyluracil (cm^6u), 6-methyluracil (m^6u), and 5-methyluracil (m^5u).

The horizontal white line at the center of each of the figures denotes the origin. The symbols "+" and "-" refer to the anode and cathode, respectively.

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 5.

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.

(10) Paper Electrophoretic Characterization of 5-Carboxymethyluridylate, 5-Carboxymethyluridine, and 5-Carboxymethyluracil Derived from tRNA

The electrophoretic mobilities listed in Table VII 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

TABLE VIII. RELATIVE CHROMATOGRAPHIC MOBILITIES OF VARIOUS URACIL DERIVATIVES

Compound	System 1	System 2
	R_{Up}	R_f
Up	1.00	0.55, 0.60*
cm^5Up (yeast tRNA)	0.88	0.62, 0.66*
cm^5Up (wheat embryo tRNA)	0.86	0.62, 0.66*
m^5Up	1.12	0.45, 0.53*
ψp	0.60	0.56, 0.63*
pU	0.85	0.55
pcm^5U (wheat embryo tRNA)	0.74	0.62
U	1.25	0.51
cm^5U (yeast tRNA)	1.18	0.56
cm^5U (wheat embryo tRNA)	1.18	0.57
m^5U	1.42	0.41
ψ	0.86	0.51
u	1.25	0.43
cm^5u (yeast tRNA)	1.28	0.53
cm^5u (wheat embryo tRNA)	1.30	0.53
cm^5u (synthetic)	1.28	0.53
cm^6u	1.34	0.45
m^5u	1.50	0.34
m^6u	1.48	0.37

*Resolution into 2'- and 3'- isomers occurred in this system.

uridine and 5-methyluridine, as well as their bases, are unchanged at pH values less than 9 (see Figures 14 - 16). As expected for 5-carboxymethyluridine and its base, acquisition of a negative charge occurs over the range of pH 3 - 5 ($pK_a^{25^o}$ = 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 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.

(11) Paper Chromatographic Characterization of 5-Carboxymethyluridylate, 5-Carboxymethyluridine, and 5-Carboxymethyluracil Derived from tRNA

The chromatographic mobilities listed in Table VIII (see

FIGURE 17

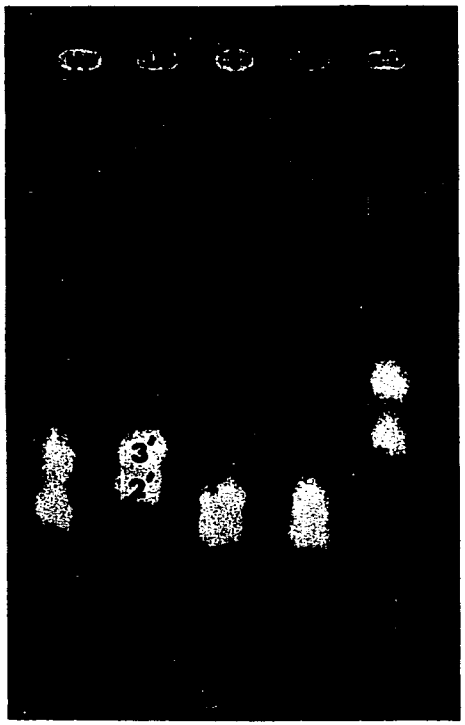


FIGURE 18

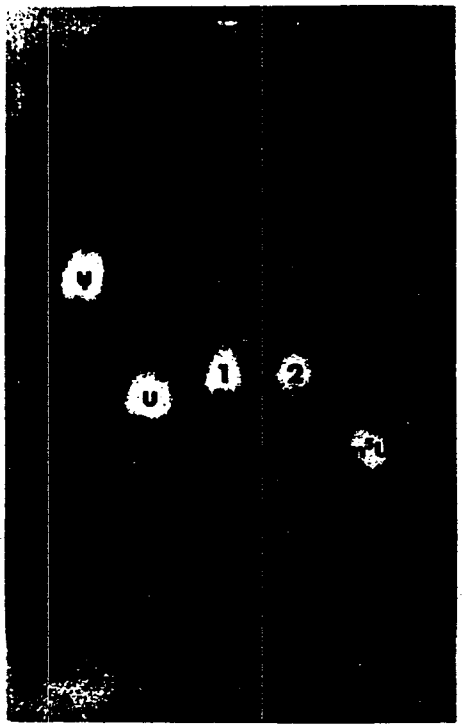


FIGURE 19

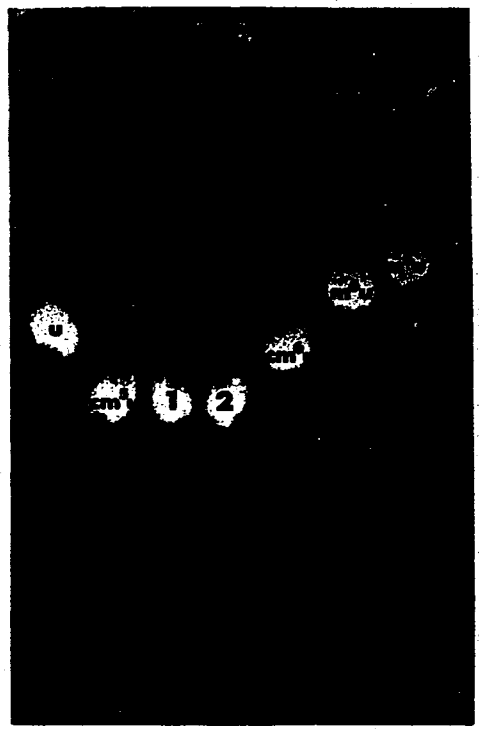


FIGURE 17

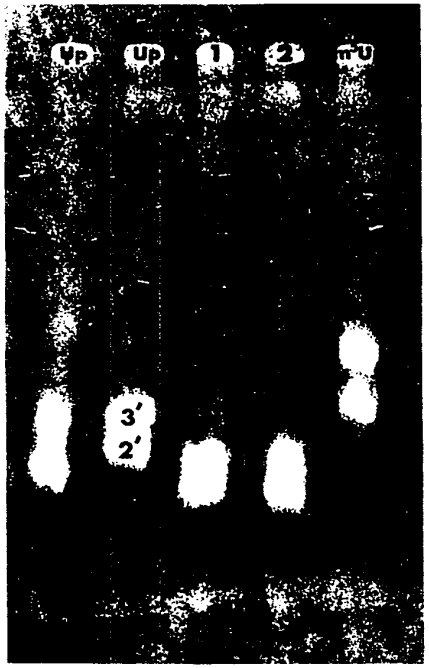


FIGURE 18

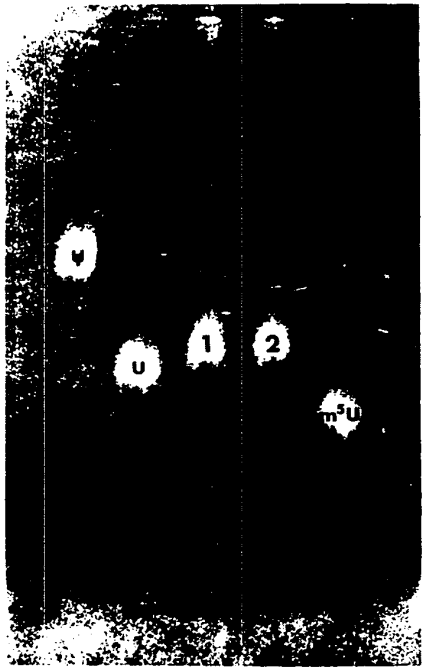


FIGURE 19

LEGEND OF FIGURE 17

An ultraviolet contact photograph depicting the paper chromatographic mobilities of various nucleoside 2'(3')-monophosphates in System 2. From left to right, the compounds are: pseudouridine 2'(3')-phosphate (ψp), uridine 2'(3')-phosphate (Up), 5-carboxymethyluridine 2'(3')-phosphate from yeast tRNA (1), 5-carboxymethyluridine 2'(3')-phosphate from wheat embryo tRNA (2), and 5-methyluridine 2'(3')-phosphate (m^5Up). The symbols "2" and "3" refer to the 2'- and 3'- isomers, respectively, of uridine monophosphate.

LEGEND OF FIGURE 18

An ultraviolet contact photograph depicting the paper chromatographic mobilities of various nucleosides in System 1. From left to right, the compounds are: pseudouridine (ψ), uridine (U), 5-carboxymethyluridine from yeast tRNA (1), 5-carboxymethyluridine from wheat embryo tRNA (2), and 5-methyluridine (m^5U).

LEGEND OF FIGURE 19

An ultraviolet contact photograph depicting the paper chromatographic mobilities of various pyrimidine bases in System 2. From left to right, the compounds are: uracil (u), synthetic 5-carboxymethyluracil (cm^5u), 5-carboxymethyluracil from yeast tRNA (1), 5-carboxymethyluracil from wheat embryo tRNA (2), 6-carboxymethyluracil (cm^6u), 6-methyluracil (m^6u), and 5-methyluracil (m^5u).

The chromatograms shown at the left were developed by the descending technique, so that the origin is at the top of each photograph.

also Figures 17-19) show that the 5-carboxymethyl derivatives of uridylate, uridine, and uracil migrate more slowly than the corresponding 5-methyl derivatives in a relatively non-polar 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 non-polar 5-methyl derivatives of uracil.

The 5-carboxymethyluridylate from alkali hydrolysates of tRNA partially resolves into two spots when chromatographed in System 2 (Figure 17), and this resolution is analagous 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.

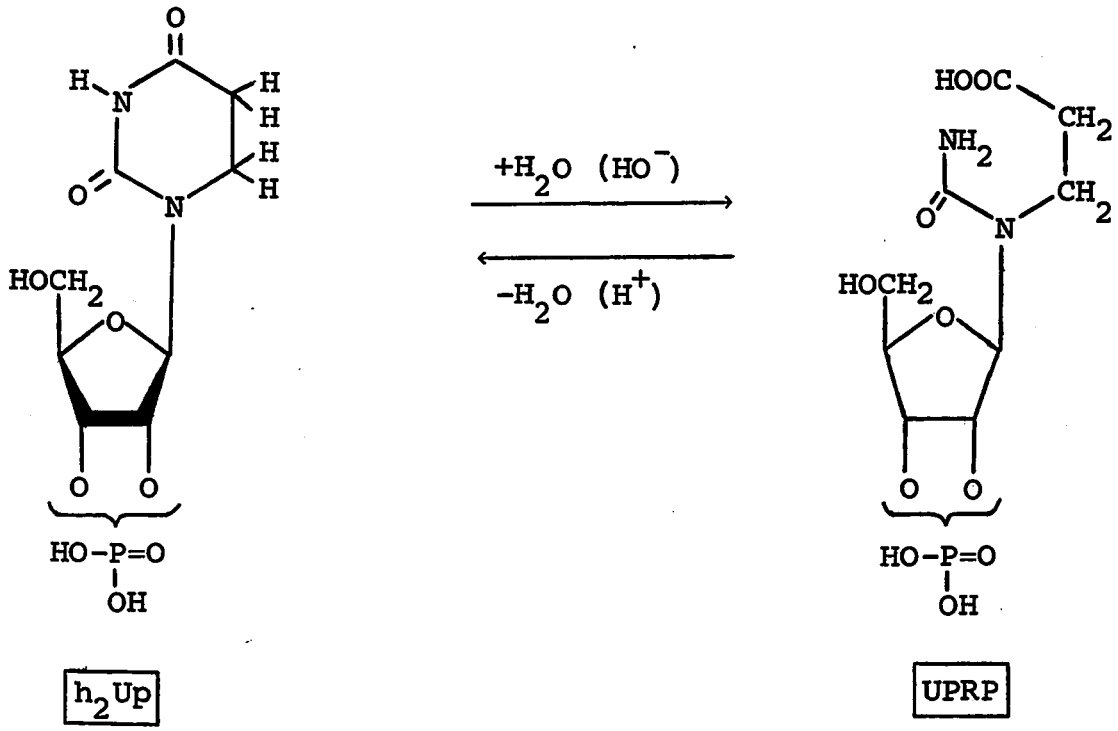
(12) Phosphorus Analysis of 5-Carboxymethyluridylate Derived from Alkali Hydrolysates of tRNA

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 per mole of nucleotide, and that after dephosphorylation, the resulting 5-carboxymethyluridine consumed ca. 2 moles of periodate per 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, Gray and Lane, 1965). However, it was found that 5-carboxymethyluridylate from yeast tRNA, which was isolated by the procedure described earlier in Part II (5) of the thesis and which was electrophoretically and chromatographically identical with the compound from wheat embryo tRNA, contained 3.8 moles of phosphorus per mole of nucleotide. From this result, it seemed likely that there was spurious contamination of the samples of 5-carboxymethyluridylate with one or more phosphorus-containing impurities which did not have appreciable ultraviolet absorbance in the 260 mμ region. This view was supported by the observation that after the above sample of 5-carboxymethyluridylate from yeast tRNA was further purified by two-dimensional chromatography (System 1 followed by System 2), the phosphorus content was reduced from 3.8 to 2.6 moles per mole of nucleotide.

After Madison and Holley (1965) reported the presence of dihydrouridylate in yeast tRNA, it became possible to speculate that at least one of the non-ultraviolet-absorbing, phosphorus-containing impurities in 5-carboxymethyluridylate specimens might be β-ureidopropionic acid N-ribosylphosphate (UPRP), the alkali-conversion product of dihydrouridylate (Scheme 3).

SCHEME 3



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, Brownlee and Barrell, 1965) and a single phospho-monoester 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.

In order to test the possibility that UPRP might be a non-ultraviolet-absorbing, phosphorus-containing impurity in preparations of 5-carboxymethyluridylate from tRNA, the 5-carboxymethyluridylate specimens were subjected to electrophoresis at several pH values between 2 and 10. After locating the ultraviolet-absorbing 5-carboxymethyluridylate, the electrophoretograms were sprayed with acidified p-dimethylaminobenzaldehyde (Fink et al, 1965), which reacts to give a yellow color with compounds having the general structure $RNHCONH_2$. In all cases, there was a yellow band overlapping, and more often, coincident with, the ultraviolet-absorbing area occupied by 5-carboxymethyluridylate. Paper chromatography in several solvent systems gave similar results, but in no case was it possible to achieve satisfactory and reproducible resolution of 5-carboxymethyluridylate from the ureido compound. In addition to the major contaminant which gave a yellow color with p-dimethylaminobenzaldehyde, and which did not cleanly resolve from 5-carboxymethyluridylate, there was evidence of at least one other compound which reacted with the Fink Reagent, but which often resolved from 5-carboxymethyluridylate during electrophoresis or chromatography on paper. Both of the compounds reacting with p-dimethylaminobenzaldehyde were present in samples of dihydrouridylate which had been treated with alkali and then stored for some time.

It is possible that the minor contaminant is β-ureidopropionic acid, formed by slow breakdown of the UPRP in 5-carboxymethyluridylate samples.

Passage of solutions of 5-carboxymethyluridylate contaminated with UPRP through a disk of charcoal (Lane and Tamaoki, 1967) was found to be the most effective means of separating the nucleotide from UPRP, since the latter compound was much less strongly adsorbed to charcoal than was 5-carboxymethyluridylate[2]. The fate of UPRP was easily monitored by the orcinol color reaction for pentose (Mejbaum, 1939), since the N-ribosyl bond in UPRP is quickly hydrolyzed in the acid environment used for the orcinol test (Cohn and Doherty, 1956), while the N-ribosyl bond in 5-carboxymethyluridylate is relatively stable under the same conditions (6 M HCl, 100°, 20 minutes). Before passage through charcoal, the 5-carboxymethyluridylate isolated from yeast tRNA contained ca. 4 moles of phosphorus per mole of 5-carboxymethyluridylate, and ca. 3 moles of pentose

---

[2]For an aqueous solution containing ca. 0.1 μmole 5-carboxymethyluridylate per ml, the best resolution was achieved at a ratio of 10 mg charcoal/μmole 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/nucleotide ratios.

(determined by the orcinol color reaction, using ribose as a standard) per mole of 5-carboxymethyluridylate. However, most of the phosphorus and orcinol-positive material passed directly through the charcoal, unadsorbed, whereas the 5-carboxymethyluridylate which adsorbed to charcoal and which was eluted with ethanolic pyridine now contained only 1.3 moles of phosphorus, and ca. 0.3 moles of pentose, per mole of 5-carboxymethyluridylate. When subjected to paper chromatography in 50% aqueous ethanol, the unadsorbed material (conversion products of dihydrouridylate) gave the expected intense yellow color reaction with p-dimethylaminobenzaldehyde, whereas the adsorbed material (5-carboxymethyluridylate) gave only a very faint reaction with p-dimethylaminobenzaldehyde.

The conclusion that UPRP contaminates samples of 5-carboxymethyluridylate has been further verified by treating the different specimens with hot alkali, after the procedure of Magrath and Shaw (1967), which gives a 70% yield of β-alanine from UPRP. The yield of β-alanine from the original 5-carboxymethyluridylate specimen from yeast tRNA was 2.3 moles per mole of 5-carboxymethyluridylate, and when compared with the results of the phosphorus analysis on this same sample of 5-carboxymethyluridylate, this value indicated that at least 80% of the phosphorus-containing contaminant could be UPRP[3]. The yield

---

[3]The remainder (20%) of the contaminant phosphorus could be in the form of inorganic phosphate or ribose phosphate, since there

TABLE IX. 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]		5-Carboxymethyluridine[b]
	This Report	Magrath and Shaw (1967)	
Yeast tRNA	3.0[c]	3.6	0.34
E. coli tRNA	2.2	2.2	n.d.
Wheat embryo tRNA	1.9	---	0.15
Yeast rRNA	---	0.02 - 0.04	n.d.
E. coli rRNA	---	0.02 - 0.04	n.d.
Wheat embryo rRNA	n.d.	---	n.d.

[a] Estimated by the procedure of Magrath and Shaw (1967)

[b] Estimated as 5-carboxymethyluridylate recovered from alkali hydrolysates

[c] This values refers specifically to *brewers'* yeast tRNA

n.d. = not detectable

appears to be some breakdown of UPRP during storage in aqueous solution. Any β-ureidopropionic acid formed by such breakdown of UPRP would be relatively resistant to further conversion to β-alanine (Magrath and Shaw, 1967), and so the total amount of contaminant, as estimated by β-alanine analysis, would be somewhat lower than the amount estimated by phosphorus analysis.

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of β-alanine, per mole of 5-carboxymethyluridylate, was sevenfold smaller in the case of the charcoal-purified 5-carboxymethyluridylate than in the case of the unpurified 5-carboxymethyluridylate. In accord with the faint reaction with the Fink Reagent, the slight orcinol reactivity, and the phosphorus/base ratio slightly greater than unity, the small amount of β-alanine (ca. 0.3 mole per mole of nucleotide) derived from the charcoal-purified 5-carboxymethyluridylate indicated a continuing but much smaller contamination with UPRP.

Table IX 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 dihydrouridine in alkali, is relatively stable to further breakdown under the conditions used 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 section of the thesis 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 the procedure described in Part II (5) is probably all contributed by a single non-ultraviolet-absorbing contaminant, UPRP, the alkali-conversion product of dihydrouridylate.

(13) 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. Under the conditions of periodate treatment, uridine 2'(3')-phosphate was also unaffected, whereas uridine 5'-phosphate was oxidized to a product (a dialdehyde), which did not contain the *cis*-diol grouping originally present in uridine 5'-phosphate. Lack of reactivity with periodate, failure to complex with borate, and partial resolution into two spots upon paper

chromatography in System 2, are all properties shown by isomeric mixtures of 2'- and 3'-nucleotides, such as the uridine 2'- and 3'-monophosphates isolated from alkali hydrolysates of RNA.

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 behaviour is expected when a 2'(3')-ribonucleotide is converted to 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 per 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, by vol.). 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 four-fold 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).

(14) Occurrence of 5-Carboxymethyluridylate in Alkali Hydrolysates of RNA

To date, 5-carboxymethyluridylate has been isolated from the tRNA of bakers' yeast, brewers' yeast, and wheat embryo. As shown in Table IX, 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 VII and VIII illustrate that the 5-carboxymethyluridylate isolated from yeast tRNA has the same electrophoretic and chromatographic properties as that isolated from wheat embryo tRNA (see also Figure 17). 5-Carboxymethyluridylate could not be detected in alkali hydrolysates of E. coli tRNA, nor has it been found in alkali

SCHEME 4

WHEAT EMBRYO tRNA

↓ PDE

PHOSPHODIESTERASE HYDROLYSATE
(23,800 A_{260} units)

↓

FRACTIONATION ON DEAE-FORMATE ACCORDING TO NET CHARGE AT pH 7.8

Eluent (1)	Eluent (2)	Eluent (3)	Eluent (4)
↓	↓	↓	↓
Fraction 1	Fraction 2	Fraction 3	Fraction 4
(net charge = 0 to -1)	(net charge = -2)	(net charge = -3)	(net charge > -3)
390 A_{260} units (1.64 %)	23,000 A_{260} units (96.64 %)	170 A_{260} units (0.71 %)	240 A_{260} units (1.01 %)
nucleosides (mainly A); pm^1A	nucleoside 5'-monophosphates (pN)	nucleoside 2',3'(cyclic), 5'-bisphosphates (mainly pU>p and pC>p); unknowns Y_1, Y_2, and Y_3	unhydrolyzed oligonucleotide residue

---

Eluents: (1) 0.025 M Tris formate (pH 7.8); (2) 0.085 M Tris formate (pH 7.8), 7.3 M in urea; (3) 0.17 M Tris formate (pH 7.8), 6.6 M in urea; (4) 1 M pyridinium formate (pH 4.5), after washing column with water

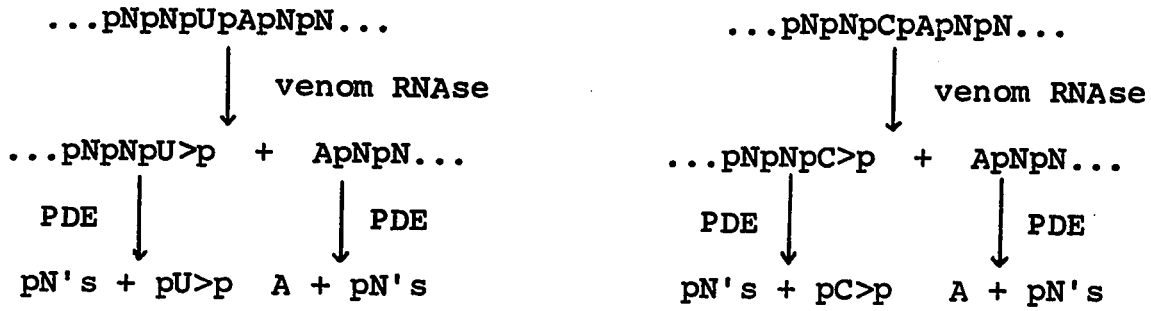
Wheat embryo tRNA (1.00 g, purified by chromatography on DEAE-cellulose) was dissolved in 100 ml of water. Fifty ml ammonium formate buffer (pH 9.2, 0.5 M in formate) were added, followed by 50 ml of a 0.03% solution of phosphodiesterase, purified from the venom of Vipera russelli as previously described (Lane, Diemer and Blashko, 1963). The moderately alkaline solution was allowed to stand at 37° for 24 hours, after which time the resulting phosphodiesterase hydrolysate was adjusted to pH 7.8 with conc. formic acid, diluted with water to reduce the formate ion concentration to 0.025 M, and applied to a 4.25 cm (i.d.) X 30 cm (h.) column of DEAE-formate, equilibrated with 0.025 M Tris formate (pH 7.8). Fraction 1 was not retained by the column, while Fractions 2 and 3 were eluted in a stepwise manner using urea-containing formate buffers of increasing ionic strength. Fraction 4 was eluted with 1 M pyridinium formate (pH 4.5), after first washing the column with water to remove salt and urea. Desalting of Fractions 2 and 3 was accomplished by re-adsorbing the compounds on columns of DEAE-formate (after diluting the fractions four-fold with water), washing the columns with water to remove Tris formate and urea, and eluting the salt-free compounds with 1 M pyridinium formate (pH 4.5), which was then removed in vacuo.

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). A recent examination of a sample of rabbit liver "sRNA", prepared by General Biochemicals according to the method of Cantoni et al (1962), has shown that 5-carboxymethyluridylate is also undetectable in alkali hydrolysates of this type of tRNA (Tumaitis and Lane, unpublished observations).

(15) Isolation of 5-Carboxymethyluridine 5'-phosphate from tRNA

If 5-carboxymethyluridine were present as the free carboxylic acid in native tRNA, then it should be possible to isolate the 5'-nucleotide of 5-carboxymethyluridine from a snake venom phosphodiesterase hydrolysate of yeast or wheat embryo tRNA. Any 5-carboxymethyluridine 5'-phosphate (pcm^5U) produced by the phosphodiesterase hydrolysis of the tRNA would be expected to be eluted from DEAE-cellulose (pH 7.8) after the other 5'-nucleotides, in the same manner as 5-carboxymethyluridine 2'(3')-phosphate is eluted after the other 2'(3')-nucleotides upon fractionation (at pH 7.8) of an alkali hydrolysate of yeast or wheat embryo tRNA (see Scheme 2). To check this possibility, wheat embryo tRNA was degraded with purified snake venom phosphodiesterase under conditions which brought about virtually complete hydrolysis of the RNA, and the hydrolysis products were then fractionated as shown in Scheme 4. It should

be noted that the phosphodiesterase preparation contained a small amount of the venom ribonuclease described by McLennan and Lane (1968). This ribonuclease has a high degree of preferential specificity for PypA bonds in ribonucleate chains, and the combined action of this endonuclease and the (primarily) exonucleolytic phosphodiesterase may be summarized as follows:



Thus, the presence of the contaminant endonuclease resulted in an increased amount of adenosine in Fraction 1, and an increased amount of pU>p and pC>p in Fraction 3 (Scheme 4). This was not a disadvantage, however, since the pU>p served as a useful marker. Its presence in Fraction 3 provided assurance that any 5-carboxymethyluridine 5'-phosphate in the hydrolysate would have been isolated by the fractionation procedure employed, since pU>p and pcm^{5}U have the same charge properties at pH 7.8.

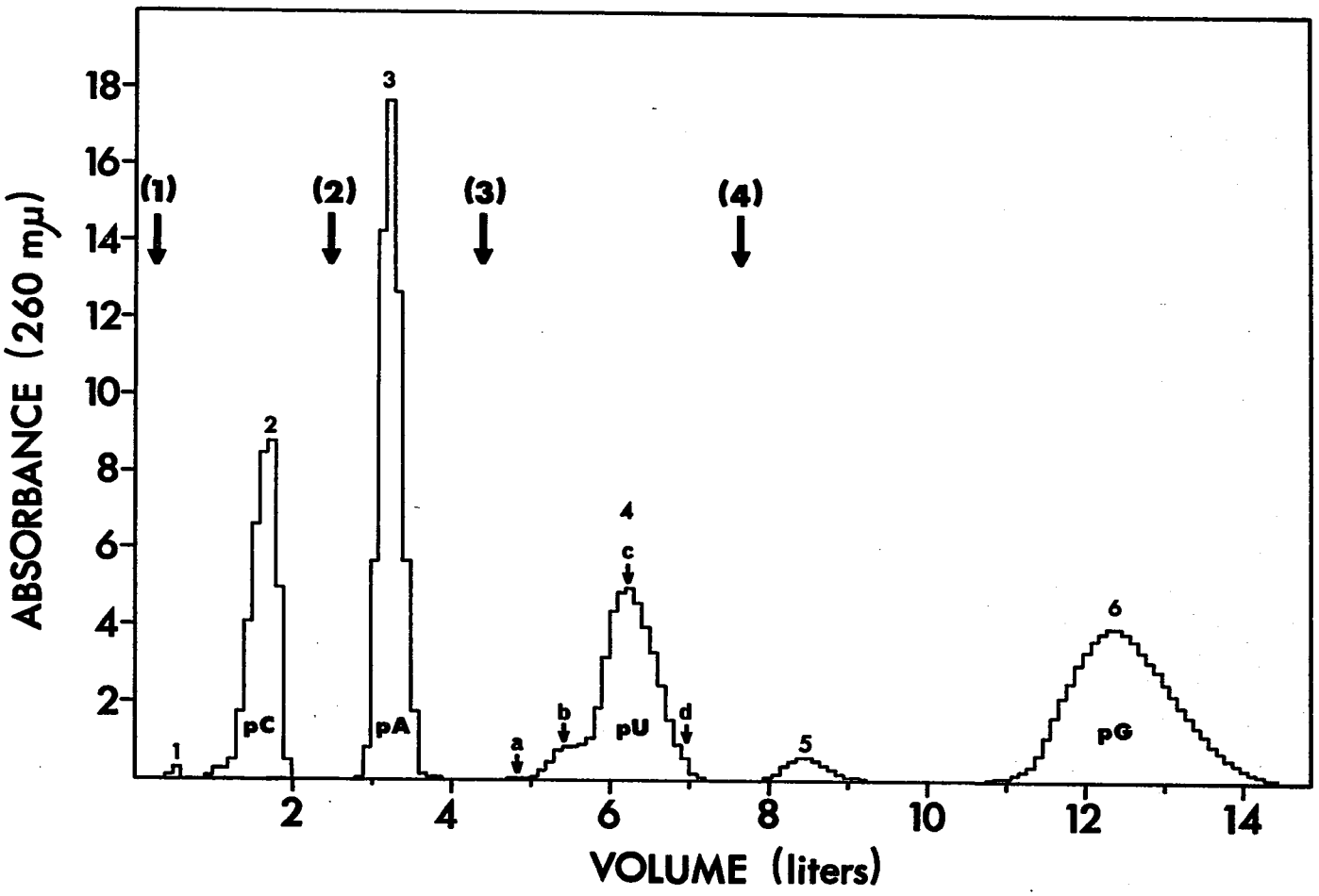
Two-dimensional chromatography (System 1 followed by System 2) of an aliquot of desalted Fraction 3 revealed no material which could have been 5-carboxymethyluridine 5'-phosphate. It was considered, however, that pU>p and pcm^{5}U

might have very similar R_f values in the two solvent systems used, so that the relatively large spot of pU>p might have contained some pcm^{5}U (the mobility of pU>p is illustrated in Figure 21). Accordingly, a second aliquot of Fraction 3 was treated with sodium hydroxide in order to convert the cyclic bisphosphates (pN>p) to the "open" forms (pNp), and the products were then fractionated on DEAE-cellulose. The "open" bisphosphates, having a net charge of -4 at pH 7.8, remained on the column during the elution of the other components with 0.17 M Tris formate (pH 7.8), 6.6 M in urea; any pcm^{5}U present would have been eluted with the 0.17 M buffer and would have thus been separated from pUp. However, when the uv-absorbing material eluted with 0.17 M Tris formate was analyzed by two-dimensional chromatography, 5-carboxymethyluridine 5'-phosphate was not detected.

It was found, however, that alkali treatment (1 M NaOH, 90 hours, room temperature) of the bulk 5'-nucleotides of Fraction 2 from a PDE hydrolysate of wheat embryo tRNA 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'-

FIGURE 20


ABSORBANCE (260 mμ)
18
16
14
12
10
8
6
4
2
(1)
(2)
(3)
(4)
1
2
3
4
5
6
a
b
c
d
pC
pA
pU
pG
2
4
6
8
10
12
14
VOLUME (liters)

LEGEND OF FIGURE 20

A diagram illustrating the resolution of 5'-nucleotides from a PDE hydrolysate of brewers' yeast tRNA by chromatography on Dowex-1-formate. A phosphodiesterase hydrolysate of yeast tRNA was subjected to a preliminary fractionation on DEAE-cellulose, as described in Scheme 4, and 22,850 A_{260} units of the isolated, desalted 5'-nucleotides (Fraction 2) were then applied to a 2.0 cm (i.d.) X 20 cm (h.) column of Dowex-1-formate, equilibrated with 0.025 M ammonium formate (pH 7.8). The adsorbed nucleotides were further fractionated by stepwise application of the following eluents: (1) 0.02 M formic acid (pH 2.75); (2) 0.2 M formic acid (pH 2.2); (3) 0.05 M formate-0.01 M formic acid (100 ml 1 M ammonium formate (pH 7.0) + 0.84 ml conc. formic acid, in 2 l.; measured pH = 4.3); (4) 0.1 M formate-0.1 M formic acid (200 ml 1 M ammonium formate (pH 7.0) + 8.4 ml conc. formic acid, in 2 l.; measured pH = 3.6). A flow rate of 8 ml/min was maintained, and 100 ml fractions were collected. Formic acid was removed *in vacuo* from Fractions 1 - 3 (the pH value of Fraction 1 was first adjusted to 4.5 with pyridine), while Fractions 4 - 6 were desalted by re-adsorbing the components on DEAE-cellulose, washing with water to remove salt, and eluting the desalted compounds with a volatile buffer, 1 M pyridinium formate (pH 4.5). Preliminary analysis of the various fractions has revealed the following distribution of 5'-nucleotides: Fraction 1 - pm^7G; Fraction 2 - pm^5C (leading edge), pC; Fraction 3 - pA, pm^6A (trailing edge); Fraction 4 - ph_2U (a), $p\psi$ (b), pU (c), pm^5U (d); Fraction 5 - methylated guanosine 5'-phosphate(s); Fraction 6 - pG.

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 VIII); (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 to a derivative electrophoretically and chromatographically indistinguishable from the 5-carboxymethyluridine produced by PME treatment of 5-carboxymethyluridine 2'(3')-phosphate.

TABLE X. YIELD OF 5-CARBOXYMETHYLURIDINE 5'-PHOSPHATE AFTER ALKALI TREATMENT OF THE "URIDINE 5'-PHOSPHATE" FRACTION FROM A DOWEX-FRACTIONATED PHOSPHODIESTERASE HYDROLYSATE OF BREWERS' YEAST tRNA

Subfraction	Composition (mole %)*			µmoles treated with alkali			µmoles nucleotides per ml of 1 M NaOH during alkali treatment	µmoles uv-absorbing material in post-mononucleotide fraction		
	pψ	pU	pm^5U	pψ	pU	pm^5U		pcm^5U	Z_1	Z_2
b	87.0	13.0	----	16.4	2.46	-----	18.9 / 3 = 6.3	0.26	0.16	----
c	1.8	92.7	5.5	1.89	95.4	5.61	103 / 3 = 34.3	0.18	----	0.25
d	----	44.7	55.3	-----	1.16	1.44	2.60 / 3 = 0.87	----	----	----
								0.44	0.16	0.25

*Fraction 4 consisted of 15.5 mole % pψ, 78.8 mole % pU, and 5.7 mole % pm^5U. Two other minor components were also present, one (presumed to be pUm) in Subfraction b, the other (presumed to be pm^1I) in Subfractions c and d. Molar extinction coefficients used: pψ, 7500; pU, 10,000; pm^5U, 8700; Z_1, 7500; Z_2, 7500 (all at 260 mµ and pH 1).

The subfractions b, c, and d refer to the early, middle, and late portions, respectively, of Fraction 4 (the "uridine 5'-phosphate" fraction) from a Dowex-fractionated PDE hydrolysate of brewers' yeast tRNA (Figure 20). The leading edge (b) of Fraction 4 was enriched in pψ, while the trailing edge (c) was enriched in pm^5U. Portions of each subfraction were dissolved in NaOH (final concentration = 1 M; volume = 3 ml) and allowed to remain at room temperature for 90 hours. The resulting "hydrolysates" were then neutralized, diluted, and adsorbed on columns of DEAE-formate. The columns were washed with 0.085 M Tris formate (pH 7.8), 7.3 M in urea, until no further ultraviolet-absorbing material was eluted. Any uv-absorbing material remaining on the columns was then removed with 1 M pyridinium formate (pH 4.5), after washing the columns with water to remove salt and urea. The uv-absorbing material eluted with pyridinium formate was analyzed by two-dimensional paper chromatography (System 1 followed by System 2), and material with the properties of 5-carboxymethyluridine 5'-phosphate was found in the alkali-treated aliquots of Subfractions b and c, but not in the aliquot of Subfraction d treated with alkali. The total yield of pcm^5U, however, was only ca. 25% of the amount expected†. Besides the presumed pcm^5U, two other compounds remained on DEAE-cellulose columns during elution with 0.085 M Tris formate (pH 7.8). One of these (Z_1) resembled pψ in that its uv spectrum displayed a bathochromic shift in alkali, but the mobility of Z_1 was greater than the mobility of pψ during paper chromatography in System 2. The other compound (Z_2) had λ_{max} = 276 mμ (acid), 275 mμ (alkali). An additional amount of Z_2 was produced after re-treatment (1 M NaOH, 40°, 48 hours) of the alkali-treated 5'-nucleotides of Subfraction c.

† Approximately 2000 μmoles of 5'-nucleotides were fractionated on Dowex-1. Since 5-carboxymethyluridine 2'(3')-phosphate constitutes 0.34 mole % of the alkali hydrolysis products of brewers' yeast tRNA, ca. (2000 X 0.0034) = 6.8 μmoles of 5-carboxymethyluridine 5'-phosphate would be expected to be produced by alkali treatment of 2000 μmoles of 5'-nucleotides from a PDE hydrolysate of yeast tRNA. Assuming that Fraction 4 contained all of the compound from which pcm^5U is derived by alkali treatment, 6.8 μmoles of pcm^5U would be expected to be produced by alkali treatment of the entire amount of Fraction 4. Since only three-tenths of each subfraction was treated with alkali, the expected yield of pcm^5U was (6.8 X 0.3) = 2.0 μmoles. The actual amount of pcm^5U found was 0.44 μmoles, or (0.44/2.0 X 100) = 22% of the expected yield.

The above results suggest that 5-carboxymethyluridine does not occur in native tRNA as the free carboxylic acid, but that the carboxyl function is blocked by an alkali-labile, neutral group (this point is discussed in further detail in Section (16)). In an attempt to elucidate the nature of this presumed blocking group, the 5'-nucleotides isolated from a PDE hydrolysate of brewers' yeast tRNA were fractionated on Dowex-1-formate, using the elution scheme described by Cohn (1960) for the fractionation of the 2'(3')-nucleotides derived from an alkali hydrolysate of RNA. The resulting elution profile is shown in Figure 20.

The 5'-nucleotides of pseudouridine, uridine, and 5-methyluridine were found to elute in the early, middle, and late portions, respectively, of Fraction 4. Each of these subfractions was treated with alkali (1 M NaOH, room temperature, 90 hours) and then fractionated on DEAE-cellulose according to net charge at pH 7.8, in order to isolate any 5-carboxymethyluridine 5'-phosphate produced by the alkali treatment. In the case of the alkali-treated subfractions b and c, a compound having the uv spectral properties and chromatographic mobility of 5-carboxymethyluridine 5'-phosphate remained on the column after the elution of the other 5'-nucleotides (Table X). However, the total amount of this presumed 5-carboxymethyluridine 5'-phosphate isolated was only ca. 25% of the amount expected, based on the quantity of 5-carboxymethyluridine 2'(3')-phosphate found in alkali

hydrolysates of brewers' yeast tRNA. When the alkali-treated fractions were re-treated with 1 M NaOH at a higher temperature (40°), no additional 5-carboxymethyluridine 5'-phosphate was found, indicating that the initial low yield was not due to incomplete conversion of the blocked carboxyl function to the free acid form. The discrepancy may relate to the fractionation procedure employed for the resolution of the 5'-nucleotides, since acidic eluents were used over a period of several days. It is possible that the group which is presumed to block the carboxyl function of 5-carboxymethyluridine 5'-phosphate was slowly hydrolyzed in the course of the column chromatography, and that the resulting free 5-carboxymethyluridine 5'-phosphate chromatographed in another fraction (e.g., Fractions 5 or 6). Alternatively, the particular sample of yeast tRNA used in this experiment may have contained a significant amount of its 5-carboxymethyluridine in the free carboxylic acid form. Further work is in progress to check these possibilities.

(16) Discussion

Following the isolation of 5-carboxymethyluridine from yeast tRNA (Gray and Lane, 1967), a second carboxymethyl-substituted nucleoside was found in yeast tRNA by Baczynskyj, Biemann and Hall (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 an aliphatic 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 carboxylate, 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. The natural occurrence of carboxylate esters has been established in the case of plant pectin where the polygalacturonate chains are partly or fully esterified with methanol (Duel and Stutz, 1958). In vivo experiments have shown that the methyl groups of pectin can be derived from L-methionine (Sato et al, 1958), and Kauss, Swanson, and Hassid (1967) have recently demonstrated that a particulate enzyme preparation from mung beans (Phaseolus aureus) contains a transferase capable of transferring the methyl group of S-adenosylmethionine to the polygalacturonate present in the particles.

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, Biemann and Hall (1968) is almost 100-fold smaller than the yield of 5-carboxymethyluridylate which was obtained from alkali hydrolysates of brewers' yeast tRNA; (2) treatment of ethyl 2-thiouracil-5-acetate 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, Hung and Jones, 1965). These observations suggest that 2- and 4-thiouracil derivatives are relatively stable to alkali, and are not converted to 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 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 transfer RNA 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% transfer RNA, but although these preparations were routinely chromatographed on DEAE-cellulose in order to remove any high-molecular weight ribosomal RNA, the chromatographically-purified yeast and wheat embryo tRNA may still have contained 5 - 10 % of 5 S rRNA, judging from the quantity of uridine released from purified tRNA upon alkali hydrolysis (Hudson, Gray and Lane, 1965; Gray and Lane,

1967). While 5 S RNA itself does not contain minor components (Brownlee, Sanger and Barrell, 1967; Forget and Weissman, 1966; 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 transfer RNA (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 polynucleotide 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 transfer RNA 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 RNA's.

III. OTHER MINOR COMPONENTS IN HYDROLYSATES OF TRANSFER RNA

(1) Introduction

Certain of the minor components of tRNA were first discovered during the sequence analysis of chemically homogeneous species of tRNA, e.g., dihydrouridine in yeast alanine-tRNA (Madison and Holley, 1965) and N^4-acetylcytidine in yeast serine-tRNA (Feldmann et al, 1966). The majority of the known minor components of tRNA, however, were first encountered during fractionation of tRNA hydrolysates, as a consequence of the high resolving power of the various column and paper chromatographic procedures employed. The isolation of a given minor component from a chemical or enzymic hydrolysate of tRNA is simplified if fractionation techniques can be adapted to the specific chemical properties of that minor component. Thus, the isolation of 5-carboxymethyluridylate from alkali hydrolysates of yeast and wheat embryo tRNA, as described in Part II, was greatly facilitated by the fact that this nucleotide has one additional negative charge, relative to other nucleotides, at pH 7.8.

Several methods have been used for the hydrolysis of tRNA, preliminary to the isolation of minor components. One of these, alkali hydrolysis, was discussed in the introduction to Part II. This method has the disadvantage that certain of the minor components are known to be altered under alkaline

conditions. Thus, 1-methyladenosine undergoes rearrangement to $\underline{N}^6$-methyladenosine (Elion, 1962); 7-methylguanosine is converted to 2-amino-4-hydroxy-5-methylformamido-6-ribosyl-aminopyrimidine, which may undergo further cleavage in alkali to give 2,6-diamino-4-hydroxy-5-methylformamidopyrimidine (Haines et al, 1962; Jones and Robins, 1963); $\underline{N}^4$-acetylcytidine is hydrolyzed to cytidine and acetate (Feldmann et al, 1966); and dihydrouridine undergoes ring cleavage to give β-ureido-propionic acid N-riboside (Batt et al, 1954; Green and Cohen, 1957; Cohn and Doherty, 1956). When a minor component is isolated from an alkali hydrolysate of tRNA, therefore, care should be taken to establish whether or not the chemical structure of the component has been changed during the hydrolysis of the tRNA. In the case of 5-carboxymethyluridylate, for example, the results presented in Part II (15) indicate that while the free carboxylic acid form is found in alkali hydrolysates of yeast and wheat embryo tRNA, the carboxyl group is actually blocked when the component is part of native tRNA, becoming deblocked only upon alkali hydrolysis.

Hydrolysis of ribonucleates by specific enzymes, another generally-used method for the degradation of RNA, may be carried out under much milder conditions than those employed for chemical hydrolysis, thus minimizing structural alteration of labile minor components. For example, use of a combination of snake venom (containing phosphodiesterase) and bacterial

FIGURE 22

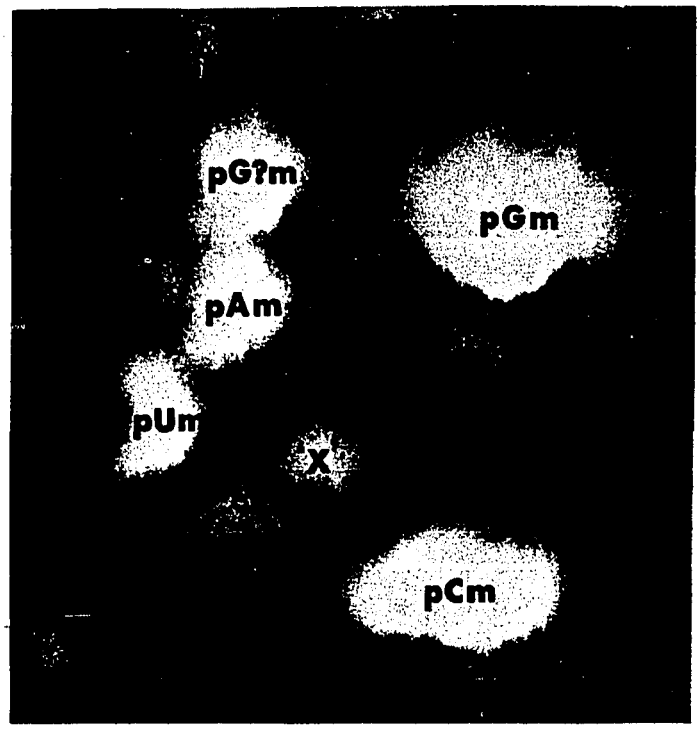


FIGURE 21

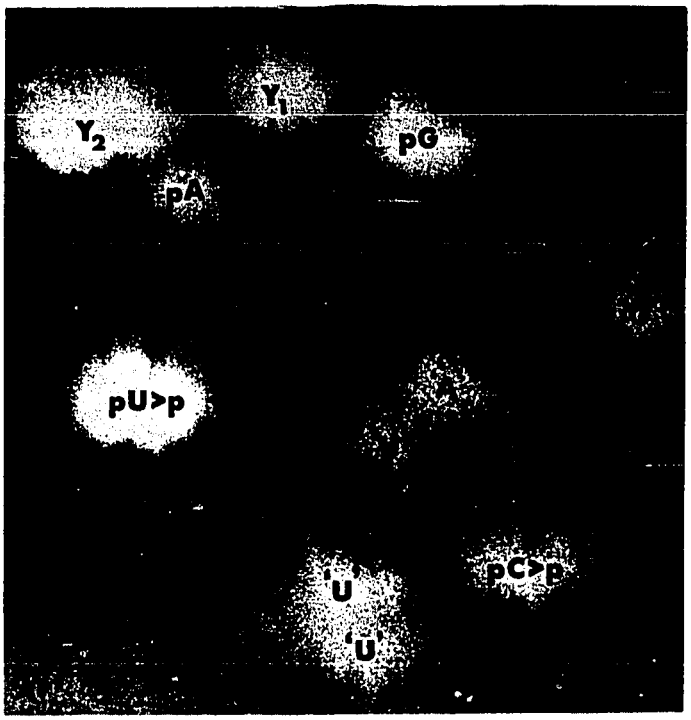


FIGURE 22

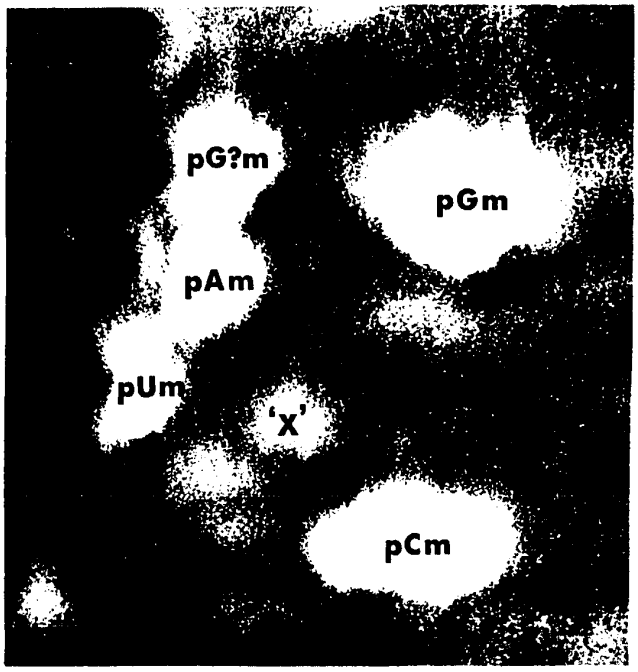
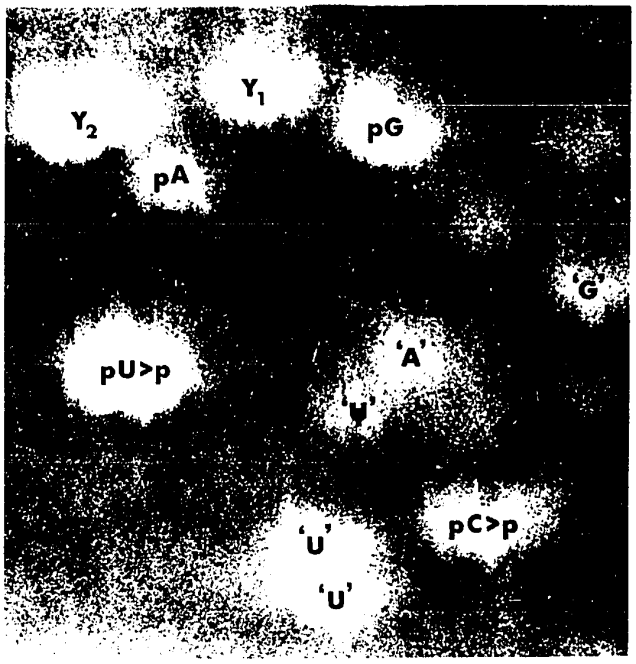


FIGURE 21



LEGEND OF FIGURE 21

An ultraviolet contact photograph depicting the two-dimensional paper chromatographic resolution of the components of Fraction 3 isolated from a PDE hydrolysate of brewers' yeast tRNA (see Scheme 4 for details of the PDE hydrolysis of tRNA and fractionation of the products). The areas marked 'G', 'A', 'ψ', and 'U' had ultraviolet absorption spectra resembling those of guanosine, adenosine, pseudouridine, and uridine, respectively. The presence of a small amount of pA and pG in this fraction is due to trailing of these compounds from the preceding fraction (Fraction 2).

LEGEND OF FIGURE 22

An ultraviolet contact photograph depicting the two-dimensional paper chromatographic resolution of the $Q^{2'}$-methylnucleoside 5'-phosphates (Fraction 2, Scheme 5) isolated from a whole venom hydrolysate of brewers' yeast tRNA. The area marked 'X' had an ultraviolet absorption spectrum similar to that of 5-substituted uridine derivatives such as 5-methyluridine and 5-carboxymethyluridine.

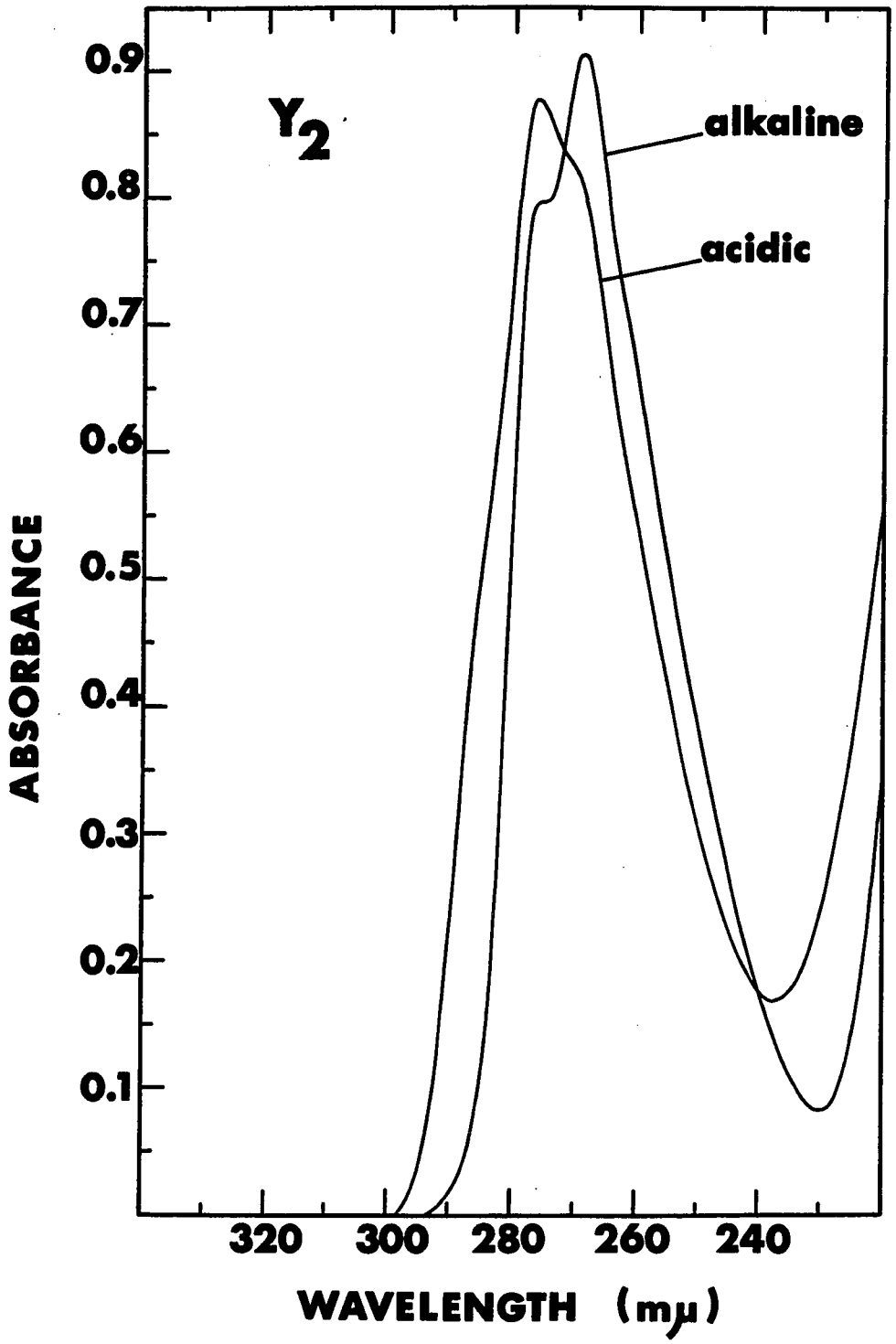
Two-dimensional chromatography was effected with System 1 in the first dimension (developed right to left in relation to the photographs), and with System 2 in the second dimension (developed top to bottom in relation to the photographs), so that the origins (not shown) were located at the upper right-hand corners of two-dimensional chromatograms.

phosphomonoesterase results in the breakdown of tRNA to its constituent nucleosides, which may then be separated by partition chromatography on Celite columns (Hall, 1965). In the present study, purified snake venom phosphodiesterase has been used to degrade tRNA to its constituent 5'-nucleotides. In addition to the mild reaction conditions, this latter hydrolytic method has the advantage that the resultant 5'-nucleotides are readily fractionated on anion-exchangers, such as Dowex-1 or DEAE-cellulose. Several compounds, as yet unidentified, have been detected during such fractionations of phosphodiesterase hydrolysates of yeast and wheat embryo tRNA, and some of their characteristics are presented in this part of the thesis.

(2) Unknowns Y_1, Y_2, and Y_3

During chromatography of a ribonuclease digest of purified yeast tRNA on DEAE-cellulose at pH 7.8, in the presence of 7 M urea, Bell, Tomlinson and Tener (1964) observed five major peaks of uv-absorbing material, corresponding to mono-, di-, tri-, tetra-, and pentanucleotides, in order of elution from the column. Immediately after each of these major peaks, a minor peak was evident, and these minor peaks were found to contain a series of unidentified compounds, which were designated Y_1, Y_2, and Y_3. These compounds did not appear to be simply contaminants, as they were not detected when undigested

FIGURE 23


Y2
alkaline
acidic
ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
320
300
280
260
240
WAVELENGTH (mμ)

LEGEND OF FIGURE 23

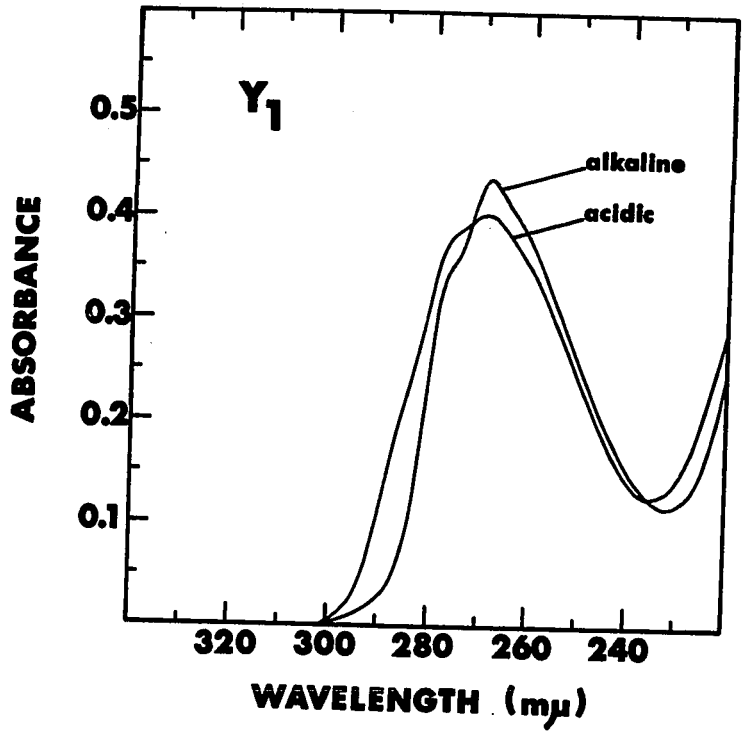
Ultraviolet absorption spectra of Unknown Y_2 isolated from a PDE hydrolysate of wheat embryo tRNA. The compound was eluted from a two-dimensional chromatogram (similar to that depicted in Figure 21) in 0.1 M HCl, and the acidic spectrum was obtained by recording the spectrum of 1 ml of this eluate against 1 ml of a 0.1 M HCl eluate of an adjacent blank area from the chromatogram (the area containing the compound and the area used for the blank were eluted in the same volume of HCl). The alkaline spectrum was determined after addition of 50 µl of 10 M NaOH to 1 ml of the acidic solutions used for determination of the acidic spectrum. λ_{max} = 276 mµ (acidic), 269 mµ (alkaline). Spectra were recorded on a Bausch and Lomb Spectronic 505 spectrophotometer.

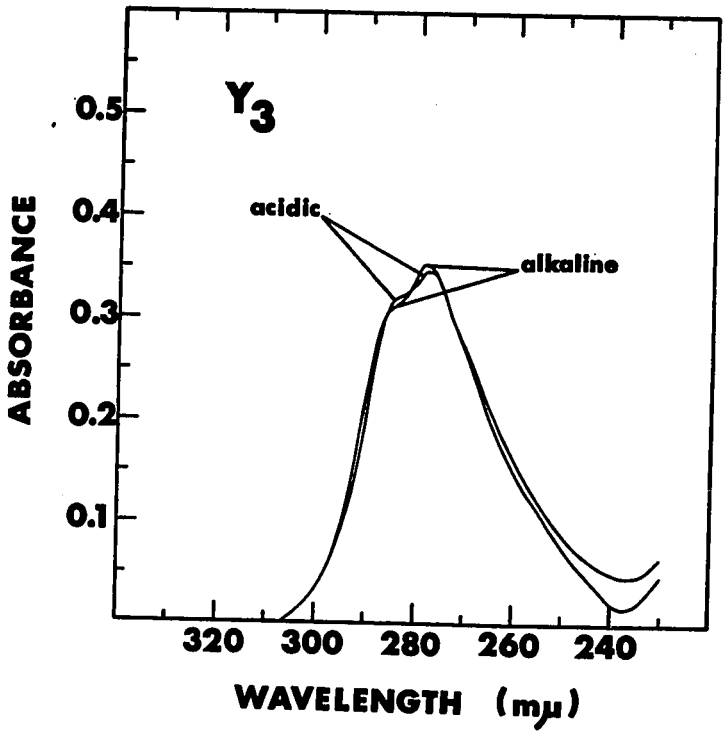
samples of the minor peaks were subjected to chromatography or electrophoresis; they were released, however, upon treatment of the minor peaks with alkali or crude snake venom phosphodiesterase. The authors stated that the unknown compounds had ultraviolet spectra bearing a slight resemblance to nicotinamide mononucleotide, and the uv absorption spectra of Y_2 were presented.

In the course of the present work, during the search for 5-carboxymethyluridine 5'-phosphate in phosphodiesterase hydrolysates of yeast and wheat embryo tRNA (Part II (15)), a series of compounds similar to those described by Bell et al was encountered. As indicated in Scheme 4, these compounds were eluted from DEAE-cellulose after the 5'-nucleotides, appearing in Fraction 3, along with the nucleoside 2',3'(cyclic), 5'-bisphosphates. The two-dimensional chromatographic separation of the components of Fraction 3 from a PDE hydrolysate of yeast tRNA is shown in Figure 21, illustrating the relative chromatographic mobilities of the unknown compounds. Trace amounts of Unknown Y_3, which is not shown in Figure 21, were found in the area to the immediate left of Y_2 on two-dimensional chromatograms of Fraction 3 from PDE hydrolysates of wheat embryo tRNA (it has not yet been determined whether Y_3 is present in PDE hydrolysates of yeast tRNA).

The ultraviolet spectra of Y_2, Y_1, and Y_3 isolated from wheat embryo tRNA are presented in Figures 23, 24, and 25, respectively. The alkaline spectrum of Y_2 shown in Figure 23

FIGURES 24 (Y_1) AND 25 (Y_3)





LEGEND OF FIGURE 24

Ultraviolet absorption spectra of Unknown Y_1 isolated from a PDE hydrolysate of wheat embryo tRNA. The compound was eluted from a two-dimensional chromatogram (similar to that depicted in Figure 21) in 0.1 M HCl, and spectra were determined as described in Figure 23, using a Bausch and Lomb Spectronic 505 spectrophotometer. λ_{max} = 269 mμ (acidic), 268.5 mμ (alkaline)

LEGEND OF FIGURE 25

Ultraviolet absorption spectra of Unknown Y_3 isolated from a PDE hydrolysate of wheat embryo tRNA. The compound was eluted from a two-dimensional chromatogram (similar to that depicted in Figure 21) in 0.1 M HCl, and spectra were determined as described in Figure 23, using a Bausch and Lomb Spectronic 505 spectrophotometer. λ_{max} = 279 mμ (acidic), 279.5 mμ (alkaline)

is identical to that of the unknown compound Y_2 described by Bell et al, although the acidic spectrum is slightly different. This difference, however, may reflect the difference in the acid concentration at which the spectra were determined (1 N acid in the case of Bell et al, vs. 0.1 N acid in the present study). It is noteworthy that, in their general shape, the uv spectra of the "Y" compounds resemble the uv spectra of such aromatic compounds as phenol and pyridine, and in this regard the spectra of these unknowns differ from those of the heterocyclic bases of RNA.

Ultraviolet-absorbing compounds with spectra resembling those of the "Y" compounds have been found in the post-mononucleotide fractions of PDE hydrolysates of all samples of wheat embryo tRNA examined to date. Table XI gives the quantitative proportions of Y_1, Y_2, and Y_3 found in phosphodiesterase hydrolysates of two different purified samples of wheat embryo tRNA, and it can be seen that the amount of each of the unknowns was approximately the same in each hydrolysate. In addition, the proportions of Y_1 and Y_2 isolated from yeast tRNA were similar to the proportions of Y_1 and Y_2 isolated from wheat embryo tRNA. The reproducibility with which these unknowns can be isolated from tRNA, as well as the high degree of purity of the tRNA preparations, argues against the possibility that these compounds are simply contaminants of the RNA (e.g., impurities in the phenol used for extraction of the RNA). In this regard, it is significant that no "Y" compounds have

TABLE XI. QUANTITATIVE PROPORTIONS OF UNKNOWN COMPOUNDS Y_1, Y_2, AND Y_3 IN PHOSPHODIESTERASE HYDROLYSATES OF YEAST AND WHEAT EMBRYO tRNA

Unknown	Yeast tRNA	Wheat Embryo tRNA	
		Thatcher 13	Thatcher 21
Y_1	0.12	0.11	0.07
Y_2	0.24	0.17	0.17
Y_3		0.02	+

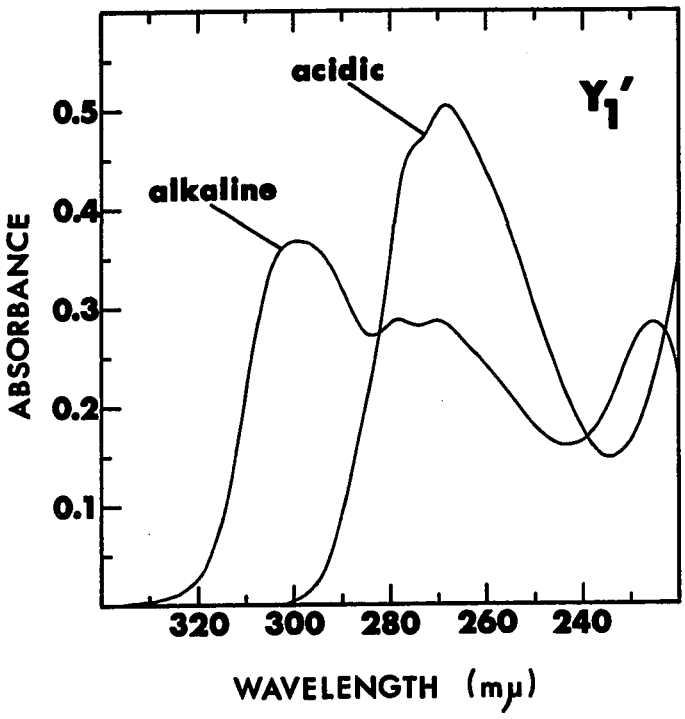
(The symbol "+" indicates that the compound was present, but that the absolute amount was not determined.)

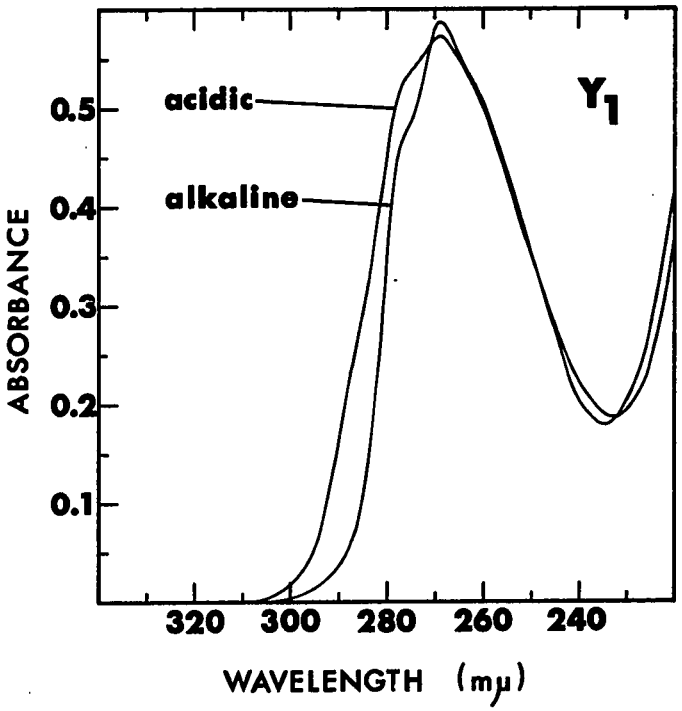
Values are listed as moles/100 moles of total nucleotides, assuming that the unknown compounds have approximately the same molar extinction coefficient as the "average" of the nucleotides in a hydrolysate of tRNA (ca. 11,000 at 260 mμ)

been found in the post-mononucleotide fractions of PDE hydrolysates of wheat embryo 18 S + 28 S ribosomal RNA.

In one experiment, after resolution of a PDE hydrolysate of wheat embryo tRNA on DEAE-cellulose at pH 7.8, Fraction 4 (Part II (15)) was found to contain not only the expected oligonucleotide residue (which accounted for ca. 90% of the uv absorption of this fraction), but also a "Y" compound. The uv spectrum of this compound (Figure 26) and its chromatographic behaviour on paper in Systems 1 and 2 suggested that it was a residual amount of Y_1 which had not been completely eluted from the column with the 0.17 M Tris formate eluent. An aliquot of this particular fraction was treated with a combination of whole snake venom and bacterial alkaline phosphatase, in order to hydrolyze the oligonucleotide residue, and the products were fractionated on DEAE-cellulose at pH 7.8. The uv-absorbing material which did not adsorb to the column consisted of nucleosides, derived from the oligonucleotide residue, while a compound designated Y_1' (since it appeared to be a derivative of Y_1) remained on the column and was eluted with 1 M pyridinium formate (pH 4.5). The uv absorption spectra of Y_1' are shown in Figure 27, and a comparison with Figure 26 shows that the acidic spectra of Y_1' and Y_1 are identical, while the alkaline spectra are markedly different. This difference was initially interpreted as being due to the alteration of the chromophore of Y_1 by treatment with whole venom-bacterial phosphomonoesterase, but a

FIGURES 26 (Y_1) AND 27 (Y_1')





LEGEND OF FIGURE 26

Ultraviolet absorption spectra of unknown "Y" compound (presumed to be Y_1) found in the "oligonucleotide fraction" (Fraction 4) after chromatography of a PDE hydrolysate of wheat embryo tRNA on DEAE-cellulose (see Scheme 4). The compound was eluted from a two-dimensional chromatogram in 0.1 M HCl, and spectra were determined as described in Figure 23, using a Bausch and Lomb Spectronic 505 spectrophotometer. λ_{max} = 269 mμ (acidic), 269 mμ (alkaline)

LEGEND OF FIGURE 27

Ultraviolet absorption spectra of Unknown Y_1', the product obtained after treatment of (presumed) Unknown Y_1 (Figure 26) with a mixture of whole snake venom and PME. The compound was eluted from a two-dimensional chromatogram in 0.1 M HCl, and the acidic spectrum was determined as described in Figure 23, using a Bausch and Lomb Spectronic 505 spectrophotometer. The alkaline spectrum was determined after addition of 100 μl of 10 M NaOH to 1 ml of the acidic solutions used for determination of the acidic spectrum. λ_{max} = 268.5 mμ (acidic), 299 mμ (alkaline)

re-examination of Y_1 has shown that the untreated compound assumes the same alkaline spectrum shown in Figure 27 at hydroxide ion concentrations greater than that used for the determination of the alkaline spectrum of Y_1 shown in Figure 26. This marked change in the alkaline spectrum of Y_1 at very high concentrations of hydroxide ion does not appear to be due to an alkali-induced chemical change in the molecule, since the alkali-dependent spectral changes are reversible upon addition of acid. It would seem, instead, that Y_1 and Y_1' contain a dissociable group which ionizes only under strongly basic conditions, and whose ionization markedly affects their ultraviolet absorption spectra.

The chromatographic mobility of Y_1 was substantially altered by treatment of the compound with whole venom plus phosphomonoesterase. In System 1, for example, Y_1 had an R_f value of 0.17, whereas Y_1' had an R_f value of 0.69. This marked increase in mobility in a relatively non-polar solvent suggests that the enzyme treatment resulted in the removal of polar group(s) (e.g., phosphate group(s)) from Y_1.

Acid hydrolysis of Y_1' (2 A_{260} units in 0.4 ml constant boiling HCl in a sealed, evacuated tube; 110°; 4 hr) produced a single compound, which was identified as uracil by its chromatographic mobility and uv spectra. The amount of uracil recovered from the acid hydrolysate, however, accounted for only ca. 25% of the uv-absorbing material originally present. On the basis of this result, it is possible that Y_1' contains

SCHEME 5

YEAST tRNA

| Whole venom

WHOLE VENOM HYDROLYSATE
(6110 A_{260} units)

↓

FRACTIONATION ON DEAE-FORMATE ACCORDING TO NET CHARGE AT pH 7.8

Eluent (1)	Eluent (2)	Eluent (3)
↓	↓	↓
Fraction 1 (net charge = 0)	Fraction 2 (net charge = -2)	Fraction 3 (net charge > -2)
5850 A_{260} units (95.76 %)	56 A_{260} units (0.92 %)	230 A_{260} units (3.32 %)
nucleosides (N)	$O^{2'}$-methylnucleoside 5'-phosphates (pNm)	nucleoside "cyclic" and "open" bisphosphates (pN>p and pNp)

Eluents: (1) 0.025 M ammonium formate (pH 7.8); (2) 0.085 M ammonium formate (pH 7.8) 7.3 M in urea; (3) 1 M pyridinium formate (pH 4.5), after washing column with water

Brewers' yeast tRNA (300 mg, purified by chromatography on DEAE-cellulose) was dissolved in 30 ml water. Fifteen ml ammonium formate buffer (pH 9.2, 1 M in formate) were added, followed by 15 ml of a 0.3% solution of whole venom (Vipera russelli). The moderately alkaline solution was allowed to stand at 37° for 24 hours, after which time the resulting whole venom hydrolysate was adjusted to pH 7.8 with conc. formic acid, diluted with water to reduce the formate ion concentration to 0.025 M, and applied to a 2.0 cm (i.d.) x 20 cm (h.) column of DEAE-formate, equilibrated with 0.01 M ammonium formate (pH 7.8). Fraction 1 was not retained by the column. Fraction 2, after elution from the column, was desalted by re-adsorbing the $O^{2'}$-methylnucleoside 5'-phosphates on a column of DEAE-formate (after diluting the fraction four-fold with water), washing the column with water to remove ammonium formate and urea, and eluting the salt-free compounds with 1 M pyridinium formate (pH 4.5), which was then removed in vacuo. The two-dimensional chromatographic resolution of the desalted components of Fraction 2 is shown in Figure 22.

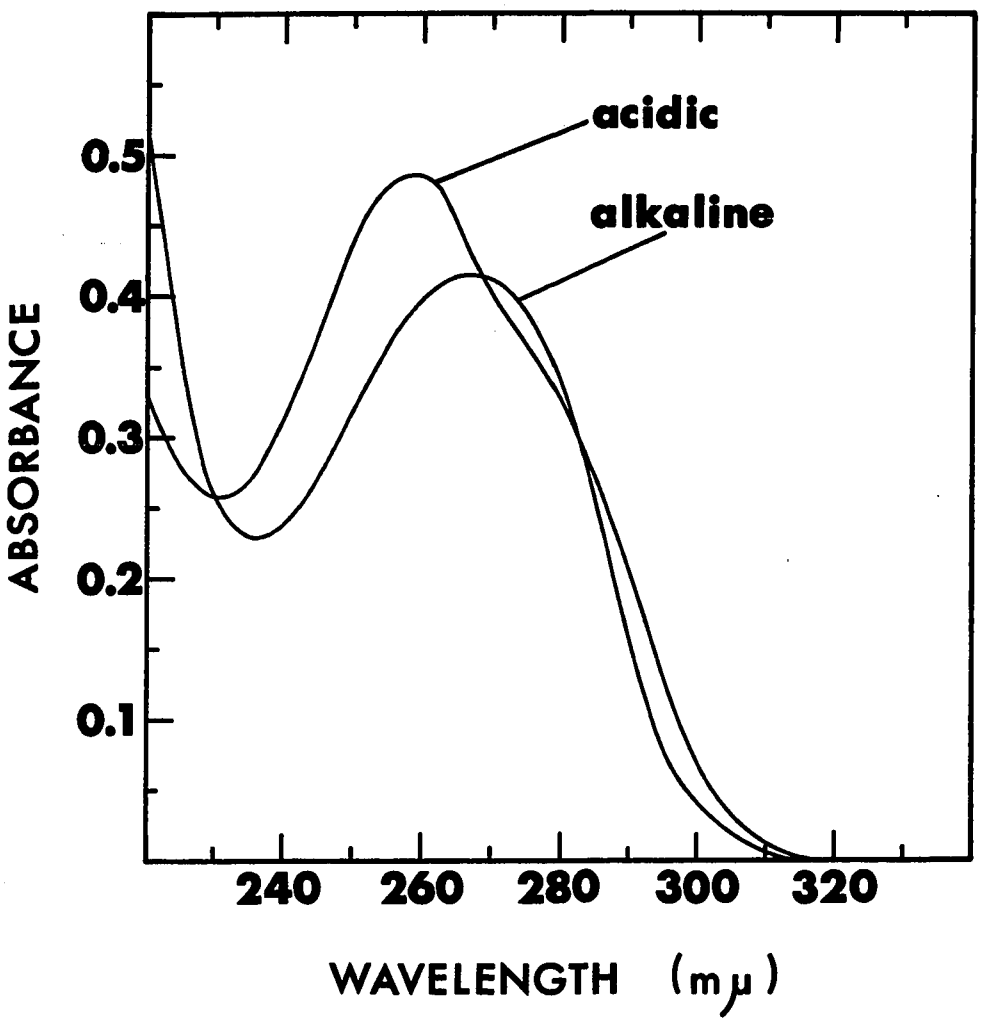
other chromophoric groups which are destroyed during acid hydrolysis.

These preliminary results on the characterization of the "Y" compounds suggest that a more detailed investigation of their origin and properties is warranted. The fractionation method by which the "Y" compounds were resolved in the present study could easily be applied to the isolation of these unknowns, on a larger scale, from phosphodiesterase hydrolysates of tRNA. A search for the "Y" compounds in phosphodiesterase hydrolysates of transfer RNA from other organisms, as well as other types of RNA, would also be informative.

(3) Unknown pG?m

The $O^{2'}$-methylnucleoside constituents of tRNA can be quantitatively isolated as part of alkali-stable dinucleotides from alkali hydrolysates of RNA, as mentioned in the introduction to Part II. The $O^{2'}$-methylnucleosides may also be isolated in the form of their 5'-nucleotides from whole venom hydrolysates of tRNA, by taking advantage of the fact that $O^{2'}$-methylnucleoside 5'-phosphates are resistant to the action of the 5'-nucleotidase present in whole venom (Honjo et al, 1964), while the other 5'-nucleotides are rapidly dephosphorylated. The resulting nucleosides can easily be separated from the $O^{2'}$-methylnucleoside 5'-phosphates by chromatography on DEAE-cellulose. The conditions for whole venom hydrolysis of

FIGURE 28


acidic
alkaline
ABSORBANCE
0.5
0.4
0.3
0.2
0.1
240
260
280
300
320
WAVELENGTH (mμ)

LEGEND OF FIGURE 28

Ultraviolet absorption spectra of Unknown pG?m, isolated from a whole venom hydrolysate of brewers' yeast tRNA (see Scheme 5 and Figure 22). The compound was purified by electrophoresis on paper at pH 1.8 (1 M formic acid), and the uv-absorbing band corresponding to pG?m was eluted in 1 ml of 0.1 M HCl for determination of the acidic spectrum. The alkaline spectrum was determined after addition of 20 µl of 10 M NaOH to the acidic eluate. Spectra were recorded on a Bausch and Lomb Spectronic 505 spectrophotometer. λ_{max} = 259 mµ (acidic), 266 - 268 mµ (alkaline)

tRNA and fractionation of the products are presented in Scheme 5.

When the $\underline{O}^{2'}$-methylnucleoside constituents of brewers' yeast tRNA were examined by the venom hydrolysis method, an unknown compound (designated pG?m) was found in the fraction containing the $\underline{O}^{2'}$-methylnucleoside 5'-phosphates (Figure 22). This unknown was found to have uv absorption spectra (Figure 28) unlike those of any of the known nucleoside components of tRNA, although the acidic spectrum of pG?m somewhat resembled, in shape, the acidic spectrum of guanosine. However, pG?m did not display the distinctive fluorescence shown by guanosine 5'-phosphate and its methylated derivatives when these latter compounds are examined on paper under uv light.

During paper electrophoresis at pH 1.8 (1 M formic acid), pG?m migrated as an anion, having a mobility of +0.46 (relative to picrate as +1.00). This was similar to the mobility of uridine 5'-phosphate in this system (+0.55), and quite different from the mobility of guanosine 5'-phosphate (-0.05). Treatment of pG?m with E. coli alkaline phosphatase gave a new compound (designated G?m) which had exactly the same mobility as uridine (-0.12) during electrophoresis at pH 1.8; under the same conditions, guanosine had a mobility of -0.64. Treatment of pG?m with 1 M NaOH at room temperature for 90 hours had no effect on the compound, as judged by the fact that its electrophoretic mobility and uv spectra were unchanged after alkaline treatment. Hydrolysis of pG?m in constant

boiling HCl for 90 minutes at 110° produced a single new compound (designated g?), which migrated as a cation during electrophoresis at pH 1.8 (g? had a mobility of -0.58, compared with a mobility of -0.64 in the case of guanosine).

The conversion of pG?m from a compound having a negative charge at pH 1.8 to a compound uncharged at pH 1.8, by treatment with PME, suggested that pG?m contained a monoester phosphate group. This conclusion was supported by the fact that pG?m was originally isolated from a fraction containing compounds having a net charge of -2 at pH 7.8. Moreover, since pG?m migrated like pU upon electrophoresis at pH 1.8, while G?m migrated like U under the same conditions, both compounds must have lacked a cationic group such as is present in adenosine, cytidine, and guanosine derivatives. However, such a cationic group seemed to be present in g?, suggesting that acid degradation of pG?m resulted in the appearance of a basic group in the product, g?.

The fact that pG?m appeared in a fraction containing only $O^{2'}$-methylnucleoside 5'-phosphates suggested that pG?m was also an $O^{2'}$-methylnucleoside 5'-phosphate, and on this basis, G?m (the product of PME treatment of pG?m) would be expected to be an $O^{2'}$-methylnucleoside. This assumption is supported by the following observations: (1) G?m did not appear to complex with borate, migrating only slightly ahead of $O^{2'}$-methyluridine, and well behind uridine, during electrophoresis at pH 9.2 in 0.025 M sodium (tetra) borate buffer;

FIGURE 29

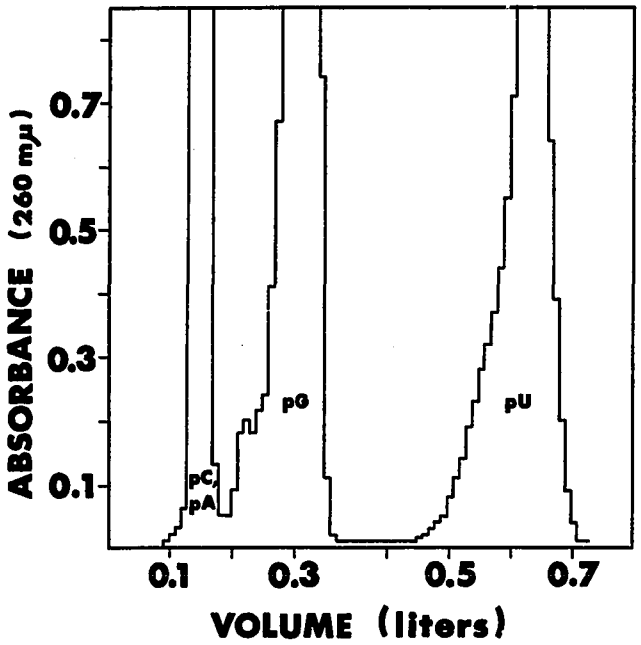
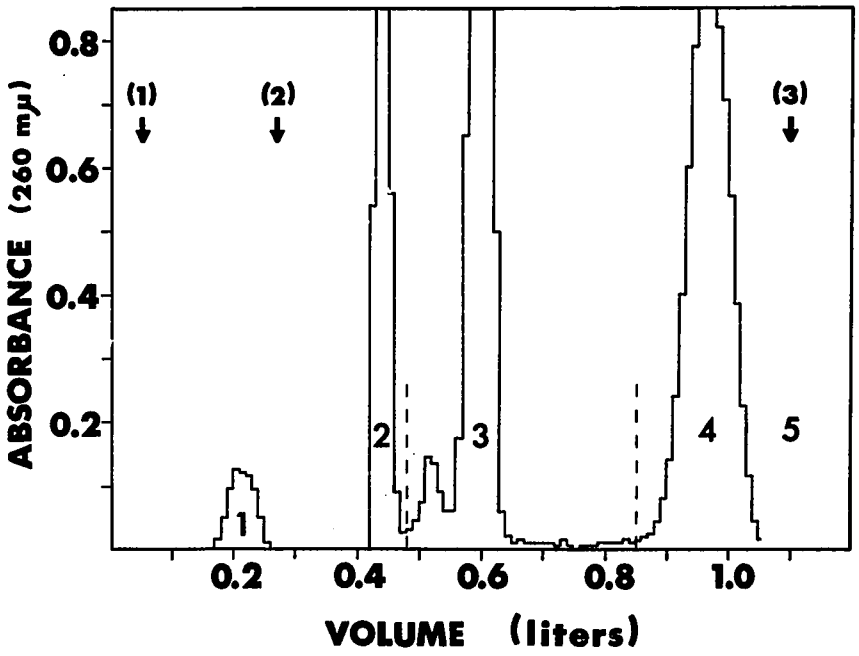


FIGURE 30



LEGEND OF FIGURE 29

A diagram illustrating the fractionation, on DEAE-cellulose at pH 1.8, of 5'-nucleotides derived from wheat embryo tRNA. The 5'-nucleotides were isolated by preliminary fractionation (at pH 7.8) of a PDE hydrolysate of wheat embryo tRNA, as described in Scheme 4. The nucleotides (564 A_{260} units) were adsorbed on a 2.0 cm (i.d.) X 30 cm (h.) column of DEAE-formate, and the column was then eluted with 1 M formic acid (pH 1.8) at a flow rate of 4 ml/min. Ten ml fractions were collected. After the elution of uridine 5'-phosphate (pU), the column was washed with water to remove formic acid, and 1 M pyridinium formate was then applied. A single compound was eluted by the pyridinium formate eluent, and this compound had ultraviolet absorption spectra very similar to those of Unknown pG?m isolated from a whole venom hydrolysate of brewers' yeast tRNA (Figure 28).

LEGEND OF FIGURE 30

A diagram illustrating the fractionation, on DEAE-cellulose at pH 1.8, of a PDE hydrolysate of brewers' yeast tRNA. The tRNA (20 mg) was hydrolyzed with purified snake venom phosphodiesterase under the conditions described in Scheme 4. The hydrolysate (containing 450 A_{260} units) was diluted to 50 ml with water and applied to a 2.0 cm (i.d.) X 30 cm (h.) column of DEAE-formate, equilibrated with 0.025 M Tris formate (pH 7.8). The eluents used, as indicated by the arrows, were: (1) water; (2) 1 M formic acid (pH 1.8); (3) 1 M pyridinium formate (pH 4.5), after washing the column with water. A flow rate of 1.4 ml/min was maintained during the elution of Fractions 1 - 4, and 10 ml fractions were collected. Fraction 1 consisted of nucleosides (mainly A), while Fraction 5 contained pU>p and a small amount of unhydrolyzed oligonucleotides. The two-dimensional chromatographic resolution of the components of Fractions 2 - 4 is shown in Figures 34 - 36. Eluents were removed *in vacuo* from the pooled column fractions, and the residual nucleotides were converted to their ammonium salts by addition and subsequent removal of excess 0.6 M ammonia. The ammonium salts were then recovered by dissolution in water, although it was found that only *ca.* 90% of the uv-absorbing material in Fraction 3 could be recovered in this manner, presumably because of the limited solubility of guanosine derivatives in water. The recovery of pm_2^2G was especially low, as shown in Figure 35. It has since been found that this problem can be avoided by using 0.6 M ammonia, rather than water, for recovery of the components of Fraction 3 and for transferal of these components to paper chromatograms.

(2) G?m did not react with periodate, indicating the absence of a vic-diol grouping in the molecule; and (3) G?m had an R_f value of 0.83 upon paper chromatography in the borate-containing solvent described by Plesner (1955) ($O^{2'}$-methylnucleosides have R_f values of 0.77 - 0.86 in this solvent, while normal nucleosides have R_f values of 0.14 - 0.30).

Since G?m appears to be an $O^{2'}$-methylnucleoside, and since the compound also appears to be stable in alkali, it is somewhat surprising that this unknown was not detected during analyses of the alkali-stable dinucleotides isolated from yeast tRNA (Gray and Lane, 1967). Assuming that G?m is actually present in transfer RNA, it is possible that this unknown occurs as part of an alkali-stable trinucleotide sequence, rather than as part of an alkali-stable dinucleotide sequence. Alkali-stable trinucleotides have been isolated from the ribosomal RNA of wheat embryo (Singh and Lane, 1964b; Lane, 1965) and yeast (Singh and Lane, 1964a; Gray and Lane, 1967), but none have so far been found in alkali hydrolysates of transfer RNA. Such trinucleotides chromatograph with nucleoside 2'(3'), 5'-bisphosphates on DEAE-cellulose at pH 7.8 (Scheme 2), and the relatively large quantity of bisphosphates (particularly pGp) present in alkali hydrolysates of transfer RNA may have obscured any alkali-stable trinucleotides present in trace amounts. This point clearly merits further investigation.

It should be noted here that a compound having uv spectra very similar to those of pG?m has been isolated from a

TABLE XII. PROPORTIONS OF THE $\underline{O}^{2'}$-METHYLNUCLEOSIDE CONSTITUENTS OF YEAST TRANSFER RNA

$\underline{O}^{2'}$-Methylnucleoside (Nm)	As pNm from Whole Venom Hydrolysate (moles/100 moles of total nucleotides)	As NmpNp from Alkali Hydrolysate* (moles/100 moles of total nucleotides)
Am	0.043	0.030
Cm	0.44	0.42
Gm	0.23	0.29
Um	0.068	0.058
Total	0.78	0.80
G?m	0.07†	

*Data taken from Gray and Lane (1967)

†Assuming a molar extinction coefficient of 11,750 (260 mμ, pH 1)

phosphodiesterase hydrolysate of wheat embryo tRNA. The compound was noted during the further fractionation of the 5'-nucleotides isolated from a PDE hydrolysate of wheat embryo tRNA (Figure 29). The unknown was the only compound to remain on the DEAE-cellulose column after the elution of the other 5'-nucleotides with 1 M formic acid (pH 1.8). It is not certain at present whether this particular unknown, isolated from wheat embryo, has the same structure as the pG?m isolated from yeast tRNA.

The amount of pG?m isolated from yeast tRNA is given in Table XII. The proportions of the four $O^{2'}$-methylnucleoside 5'-phosphates found in the whole venom hydrolysate of yeast tRNA are also listed, and the proportions of the $O^{2'}$-methyl-nucleosides of yeast tRNA, as determined by analyses of the alkali-stable dinucleotide sequences of yeast tRNA (Gray and Lane, 1967), are presented for comparison.

(4) Quantitative Analysis for Major and Minor Components of Transfer RNA

Simple methods for the detection and quantitative determination of the major and minor components of transfer RNA are of value in studies of the chemical structure of tRNA. Alkali hydrolysis of ribonucleates and resolution of the resulting nucleoside 2'(3')-phosphates is a common method for determination of the nucleotide composition of RNA, but as

FIGURE 31

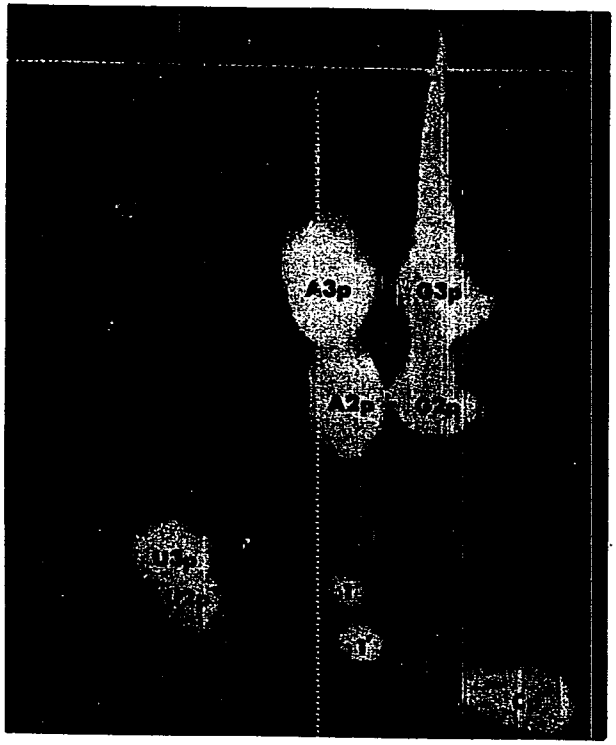


FIGURE 32

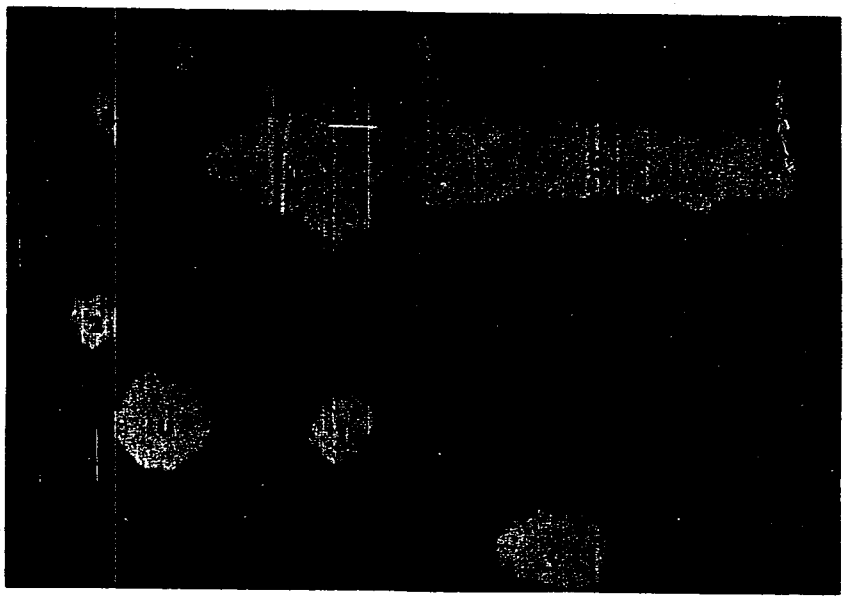


FIGURE 31

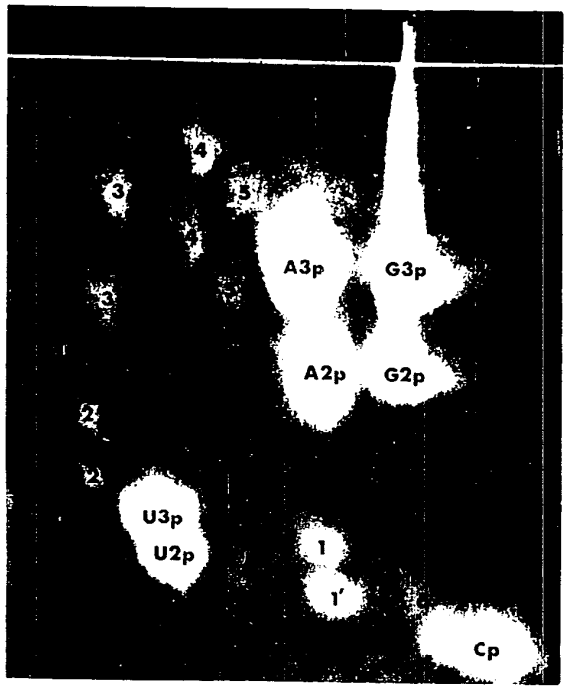


FIGURE 32

LEGEND OF FIGURE 31

An ultraviolet contact photograph depicting the two-dimensional paper chromatographic resolution of nucleoside 2'(3')-phosphates derived from brewers' yeast tRNA (the 2'(3')-nucleotides were isolated by fractionation of an alkali hydrolysate of yeast tRNA on DEAE-cellulose at pH 7.8, as described in Scheme 2). System 1 was employed in the first dimension (right to left in photograph), while System 2 was used in the second dimension (top to bottom in photograph). Note the resolution of the 2'- and 3'-nucleotide isomers in System 2 (the limited solubility of Gp in System 2 results in some trailing of this compound). The minor components were identified by their uv spectra, and the slower-moving isomer in each case was presumed to be the 3'-isomer, by analogy with the major components. The minor components detected were: ψ3p (1), ψ2p (1'), m^5U3p (2), m^5U2p (2'), m^6A3p (3), m^6A2p (3'), m_2^2G3p (4), m_2^2G2p (4'), m^1G3p (5), m^1G2p (5'), m^5Cp (6).

LEGEND OF FIGURE 32

An ultraviolet contact photograph depicting the two-dimensional paper chromatographic resolution of the nucleosides resulting from PME treatment of the 2'(3')-nucleotides from an alkali hydrolysate of brewers' yeast tRNA. The conditions employed for the dephosphorylation of nucleotides were the same as those used previously for the dephosphorylation of alkali-stable dinucleotides (Gray and Lane, 1967). The resulting nucleosides were spotted directly on ammonium sulphate-impregnated Whatman No. 1 chromatography paper, without prior removal of PME, ammonium formate, and inorganic phosphate. Two-dimensional chromatography was effected by use of System 1 in the first dimension (right to left in photograph) and System 2 in the second dimension (top to bottom in photograph). The minor components were identified by their uv spectra, and those detected were: ψ (1), m^5U (2), m^6A (3), m_2^2G (4), m^1G (5), m^2G (6), I (7), m^5C (8).

mentioned previously, several of the minor components of tRNA are labile under alkaline conditions. Furthermore, the 2'- and 3'-nucleotide isomers resolve in certain chromatographic systems, as shown in Figure 31, thereby reducing the sensitivity with which the minor components can be detected. Conversion of the nucleoside 2'(3')-phosphates to nucleosides, by treatment with E. coli alkaline phosphatase, eliminates this problem (Figure 32), and the chromatographic resolution of the resulting nucleosides is somewhat enhanced over that observed with the nucleoside 2'(3')-phosphates (especially the resolution of 5-methylcytidine from cytidine).

Since phosphodiesterase hydrolysis of tRNA can be carried out under much milder conditions than those used for alkali hydrolysis, minor components such as 1-methyladenylate and 7-methylguanylate are less susceptible to destruction. Thus, even though these components may be partially labile under the conditions of PDE hydrolysis, their presence or absence in a particular sample of tRNA can still be established with some certainty. Phosphodiesterase hydrolysis also has the advantage that no desalting of the hydrolysate is necessary prior to paper chromatographic resolution of the 5'-nucleotides, since a relatively volatile buffer (ammonium formate) is employed. About 3 - 5 mg of tRNA are sufficient for an analysis, and hydrolysates can be spotted directly on chromatography paper, with no prior pH adjustment.

FIGURE 33

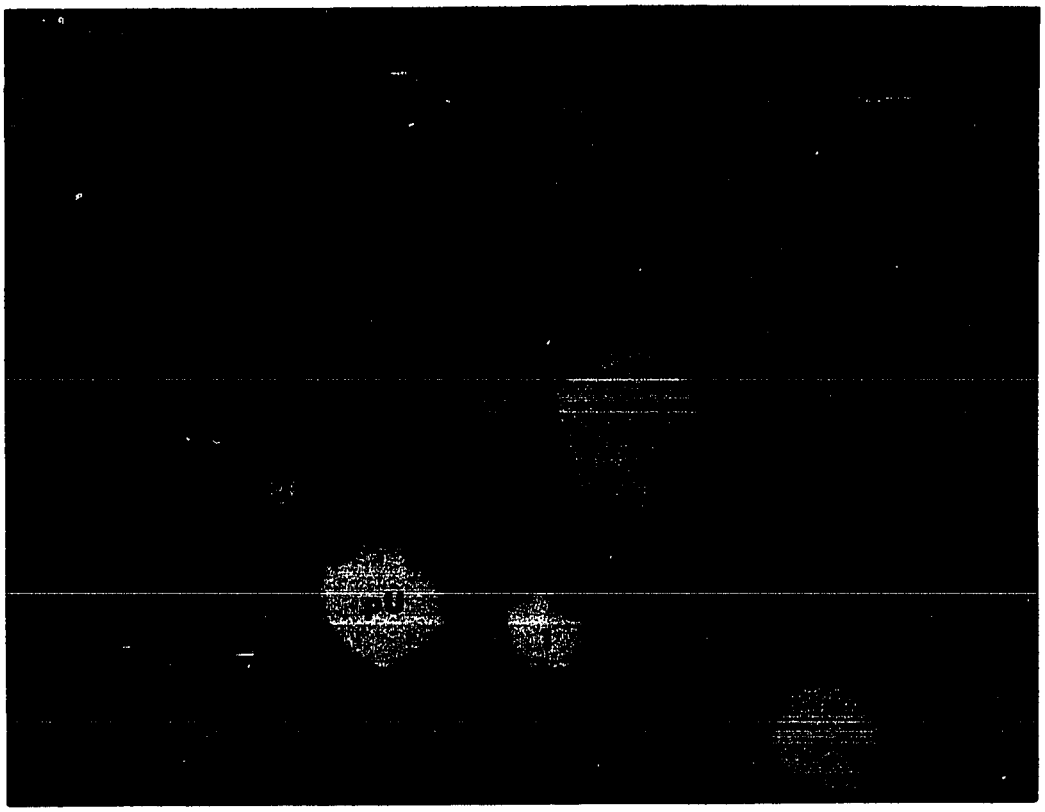


FIGURE 33

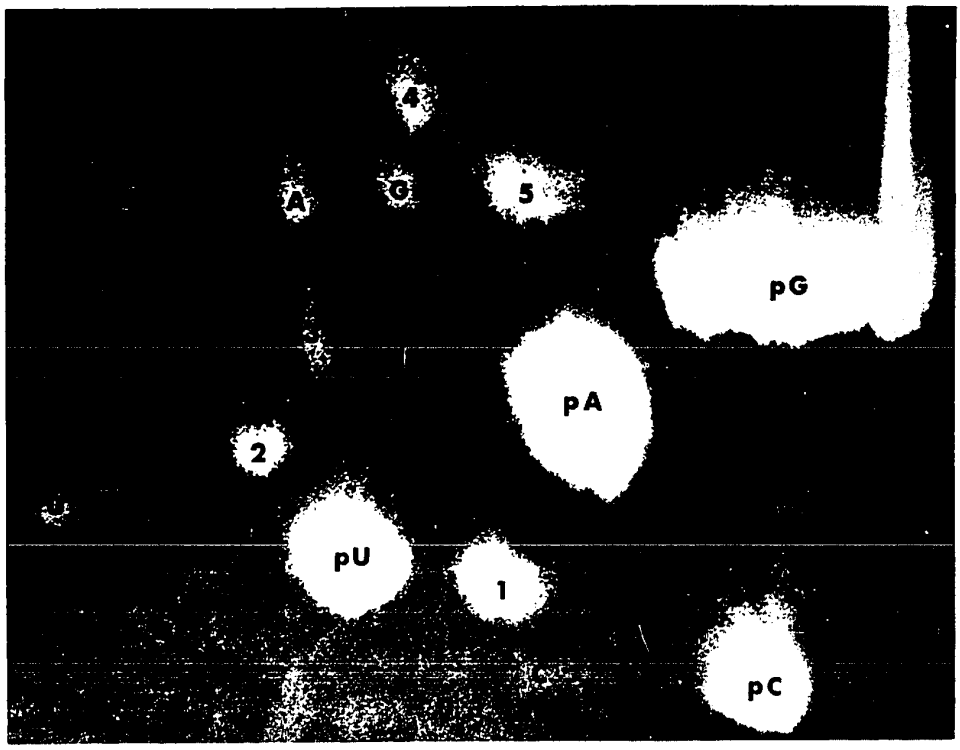
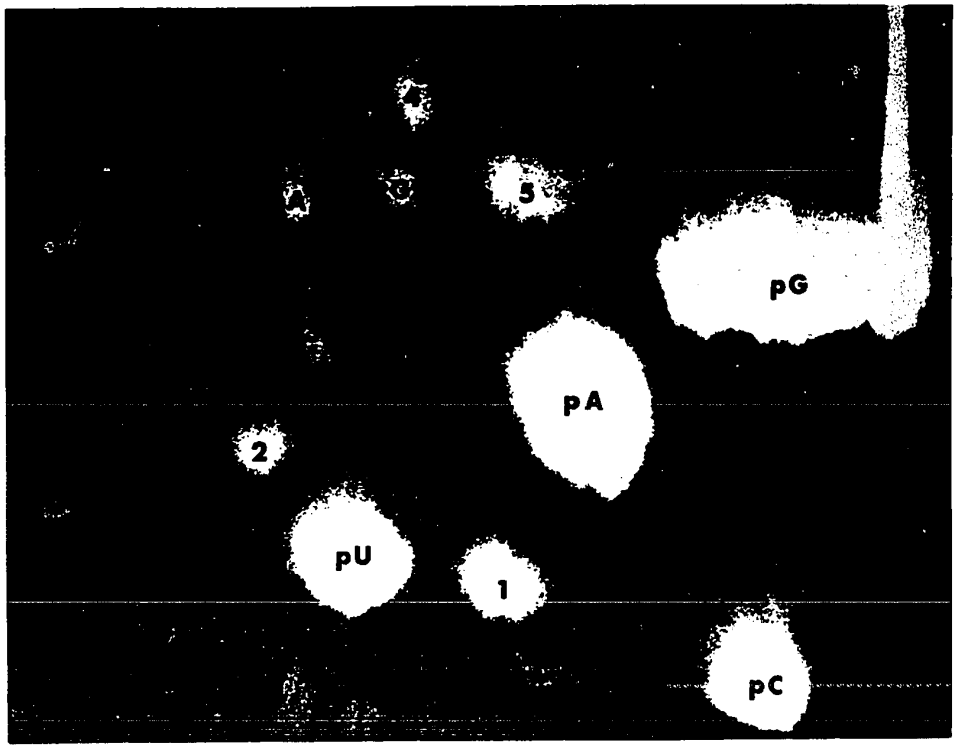


FIGURE 33



LEGEND OF FIGURE 33

An ultraviolet contact photograph depicting the two-dimensional paper chromatographic resolution of the components of a PDE hydrolysate of brewers' yeast tRNA. The tRNA was hydrolyzed as described in Scheme 4, and an aliquot (containing 75 - 100 A_{260} units) of the hydrolysate was applied directly to ammonium sulphate-impregnated Whatman No. 1 chromatography paper. The chromatogram was developed in System 1 in the first dimension (right to left in photograph) and in System 2 in the second dimension (top to bottom in photograph). The limited solubility of pG in System 2 caused this compound to trail back to the origin. The nucleotides were located on the paper chromatogram by their quenching of ultraviolet light (pG and its derivatives also displayed a distinctive bluish fluorescence), and the compounds were then quantitatively eluted from the chromatogram in 0.1 M HCl. The minor components were identified by their uv spectra, and those detected were: pψ (1), pm^5U (2), pm^6A (3), pm_2^2G (4), pm^1G (5), pm^2G (6), pm^7G (7), pm^5C (8). It should be noted that the enzyme preparation used in this particular experiment contained a trace of 5'-nucleotidase, which resulted in the appearance of detectable amounts of nucleosides in the hydrolysate. It can be seen that these nucleosides (A, C, G, U) were well separated from the other components of the hydrolysate.

TABLE XIII. PROPORTIONS OF 5'-NUCLEOTIDES IN PHOSPHODIESTERASE HYDROLYSATES OF BREWERS' YEAST AND WHEAT EMBRYO tRNA

Values are listed as mole(s)/100 moles of total 5'-nucleotides

5'-Nucleotide	Brewers' Yeast tRNA	Wheat Embryo tRNA
pA	20.0	20.1
pm^6A	0.52	0.74
pG	28.6	29.8
pm^1G	1.17	0.74
pm^7G	0.16	0.18
pm^2G	0.15	0.29
pm_2^2G	0.59	0.56
pC	25.4	26.8
pm^5C	1.36	1.39
pU	16.8	16.0
pψ	4.00	2.75
pm^5U	1.25	0.65

The proportions of the 5'-nucleotides in PDE hydrolysates of yeast and wheat embryo tRNA were determined by the procedure outlined in Figure 33. The values listed above for wheat embryo tRNA are taken from Hudson, Gray and Lane (1965) and have been re-calculated and normalized to 100% in order to include only those components which are detectable by two-dimensional chromatography of an aliquot of an unfractionated PDE hydrolysate of tRNA.

The two-dimensional chromatographic separation of the 5'-nucleotides from a PDE hydrolysate of yeast tRNA is shown in Figure 33. It can be seen that the resolution obtained is superior to that observed with either nucleoside 2'(3')-phosphates or nucleosides. Besides the four major 5'-nucleotides, at least eight minor components are easily detectable. The nucleotide compositions of yeast and wheat embryo tRNA, as determined by this method, are listed in Table XIII.

Certain of the 5'-nucleotides present in PDE hydrolysates of tRNA are not resolved by the two-dimensional chromatographic system described in Figure 33. For example, pA completely overlaps pI, while pΨ and pCm migrate together[5]. This problem

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[5] In earlier work employing a laboratory-constructed Plexiglass tank for chromatography in the second dimension (System 2), resolution of pI from pA was observed, but the separation between pm^2G and pA was poor (see Hudson, Gray and Lane, 1965, for diagram). It has subsequently been found that when a commercially-available glass tank (No. 2195 Shandon Panglas Chromatank, Consolidated Laboratories (Canada) Ltd.) is used for chromatography in the second dimension, the mobility of pA in System 2 is consistently greater than that observed in the Plexiglass tank, resulting in a complete separation of pA from the methylated derivatives of pG. At the same time, however, the increased mobility of pA causes it to completely overlap pI.

FIGURE 34

FIGURE 35

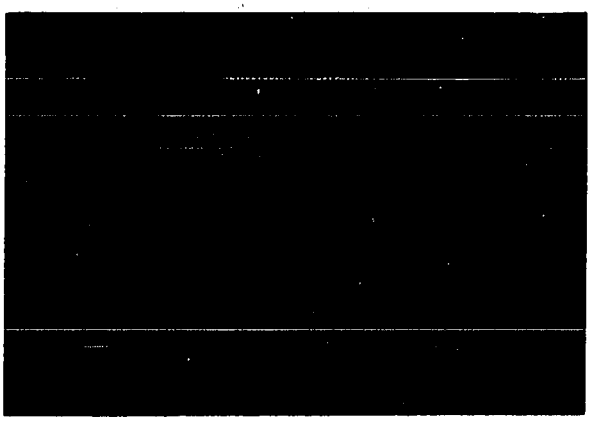


FIGURE 36

FIGURE 34

FIGURE 35

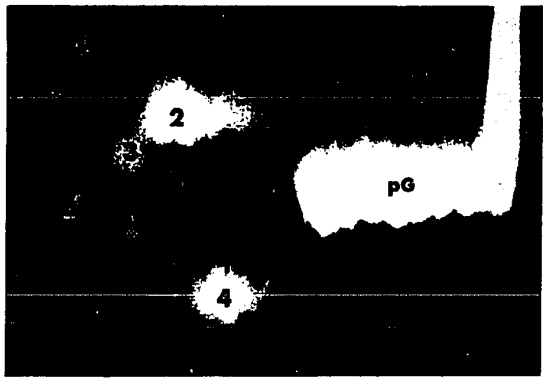
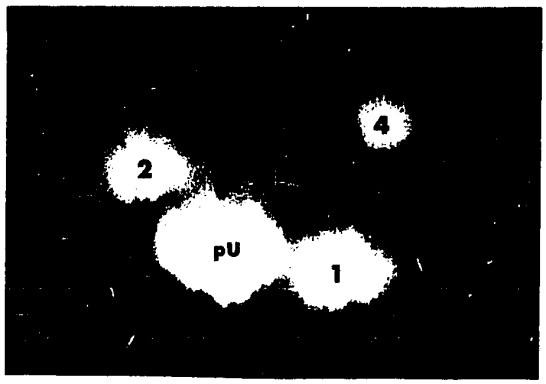


FIGURE 36



LEGEND OF FIGURE 34

An ultraviolet contact photograph depicting the two-dimensional paper chromatographic resolution of the components of Fraction 2 from a PDE hydrolysate, fractionated on DEAE-cellulose at pH 1.8, of brewers' yeast tRNA (Figure 30). Fraction 2 contained the major nucleotides pA and pC, which have a net charge of 0 at pH 1.8, as well as the minor nucleotides pm^6A (1), pCm (2), pm^5C (3), pm^1A (4), and pm^7G (5). Another uv-absorbing component (not shown in the photograph) had an R_f value of 0 in the first dimension, but migrated slightly faster than pC in the second dimension, so that this compound was found to the right of pC after two-dimensional chromatography. The unidentified component had a uv spectrum very similar to that of pC.

LEGEND OF FIGURE 35

An ultraviolet contact photograph depicting the two-dimensional paper chromatographic resolution of the components of Fraction 3 from a PDE hydrolysate, fractionated on DEAE-cellulose at pH 1.8, of brewers' yeast tRNA (Figure 30). Fraction 3 contained the major nucleotide pG, which has a net charge of ca. -0.5 at pH 1.8, as well as the minor nucleotides pm_2^2G (1), pm^1G (2), and pm^2G (3). The recovery of Fraction 3 after column chromatography was not quantitative, due to the limited solubility of guanosine derivatives in water, as explained in the Legend of Figure 30. Several unidentified compounds were detected after two-dimensional chromatography of Fraction 3, the most prominent of which (4) had an acidic uv spectrum resembling that of pG, but with a maximum at 253 mμ instead of 257 mμ. The component designated "5" is probably a trace amount of pA, trailing from the preceding fraction.

LEGEND OF FIGURE 36

An ultraviolet contact photograph depicting the two-dimensional paper chromatographic resolution of the components of Fraction 4 from a PDE hydrolysate, fractionated on DEAE-cellulose at pH 1.8, of brewers' yeast tRNA (Figure 30). Fraction 4 contained the major nucleotide pU, which has a net charge of -1 at pH 1.8, as well as the minor nucleotides pψ (1), pm^5U (2), pUm (3), and pI (4). Also present, but not shown in the photograph, were Unknown Y_2 (which had the same mobility as pU in the first dimension) and pC>p (which was located just outside the lower right-hand corner of the photograph).

can be partly overcome by a preliminary fractionation of the PDE hydrolysate (Figure 30). The 5'-nucleotides resolve into three groups upon chromatography on DEAE-cellulose at pH 1.8, and most of the overlapping compounds resolve into different fractions (e.g., pA is found in Fraction 2, whereas pI is found in Fraction 4). The components of each group can then be separated by two-dimensional paper chromatography, as shown in Figures 34 - 36. Thus, the sensitivity with which minor components can be detected is increased by the preliminary fractionation of the 5'-nucleotides. Such a procedure might prove especially useful for the detection and quantitative determination of minor components in tRNA samples consisting largely or entirely of one particular amino acid-accepting species.

SUMMARY

1. Transfer RNA was isolated from wheat embryo and was extensively purified. The purity and structural integrity of the isolated tRNA were checked by physicochemical techniques, as well as by examining the products of hydrolysis of the tRNA with alkali or purified snake venom phosphodiesterase. In addition, the biological activity of wheat embryo tRNA was tested, and the RNA was found to be a highly efficient acceptor of activated amino acids.

2. Yeast tRNA was purchased from commercial firms and was further purified. The purified RNA paralleled wheat embryo tRNA in its degree of purity and structural integrity. Pseudouridine 2'(3'),5'-bisphosphate was recovered from alkali hydrolysates of bakers' and brewers' yeast tRNA, indicating that certain (2 - 4 %) of the yeast tRNA chains contain pseudouridine at the phosphorylated (3'-linked) terminus.

3. An unknown compound, whose structure was subsequently established as 5-carboxymethyluridine 2'(3')-phosphate, was isolated from alkali hydrolysates of yeast and wheat embryo tRNA. Upon treatment of this unknown compound with E. coli alkaline phosphatase, a carboxyl-containing nucleoside, 5-carboxymethyluridine, was produced. Acid hydrolysis of this nucleoside gave a carboxyl-containing,

heterocyclic, nitrogenous base, 5-carboxymethyluracil. Evidence for the presence of a free carboxyl group in the foregoing compounds was provided by the results of chromatography on paper, and by paper electrophoresis.

4. The unknown heterocyclic base derived from transfer RNA was shown to be identical with 5-carboxymethyluracil which was synthesized by an unambiguous procedure. A comparison of the properties of the "natural" base with those of 5- and 6-carboxymethyluracil unequivocally established the C-5 position as the point of attachment of the carboxymethyl side chain in the natural base.

5. The 5'-nucleotide of 5-carboxymethyluridine was not detected in a snake venom phosphodiesterase hydrolysate of wheat embryo transfer RNA. The compound did appear, however, after treatment of the bulk 5'-nucleotides with alkali. This result indicated that 5-carboxymethyluridine does not occur as the free carboxylate in native tRNA, but that the carboxyl function is blocked by an alkali-labile group. Experiments are in progress to isolate and characterize this blocked 5-carboxymethyluridine derivative.

6. Several other unidentified compounds were encountered during the fractionation of phosphodiesterase and whole venom hydrolysates of tRNA, and the preliminary characterization of these compounds has been presented.

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