5-(4-Aminobutylaminomethyl)uracil, an Unusual Pyrimidine from the Deoxyribonucleic Acid of Bacteriophage ϕW-14†

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ABSTRACT: The DNA of bacteriophage ϕW-14 has a buoyant density of 1.666 g/cm^3 in neutral CsCl, and a melting temperature of 99.3° in standard saline–citrate, values which correspond to 4.5 and 73 mol % guanine plus cytosine, respectively. Chemical analysis shows the actual base composition to be 56 mol% guanine plus cytosine. The DNA contains five bases. About half the thymine residues are replaced with an unusual pyrimidine. The pyrimidine was purified from hydrochloric acid hydrolysates of bacteriophage ϕW-14 DNA and shown by chemical, chromatographic, and spectral analyses to be 5-(4-aminobutylaminomethyl)uracil. It was given the trivial name *N*-thyminylputrescine. The proposed structure was confirmed by chemical synthesis. The hypermodified pyrimidine is probably distributed uniformly throughout both strands of the DNA. The properties of the DNA are consistent with the covalent bonding of putrescine to some of the thymine residues.

Bacteriophage ϕW-14 is a group A type (Bradley, 1967) phage which lyses some strains of *Pseudomonas acidovorans* (Kropinski and Warren, 1970). Preliminary characterization of the DNA indicated that its buoyant density and melting temperature gave widely divergent values for the mol % guanine plus cytosine content. Such divergence often results from the presence of an unusual base in the DNA. This report describes the isolation, characterization, and synthesis of an unusual pyrimidine which only partially replaces thymine in ϕW-14 DNA.

Materials and Methods

Chemicals. 5-Hydroxymethyluracil was purchased from Mann Research Laboratories; 5-hydroxymethylcytosine from Calbiochem; 5-carboxyuracil from Aldrich Chemical Co.; 5-aminouracil from Sigma Chemical Co. 5-Carboxymethyluracil was generously donated by Dr. B. J. Lane.

Analytical Methods. All chemical analyses were made by the Alfred Bernhardt Mikroanalytisches Laboratorium, West Germany. Nuclear magnetic resonance spectra were measured with a Varian Associates MA-100 instrument equipped with audiooscillator (Model 4204A, Yokogawa-Hewlett-Packard, Tokyo, Japan) for decoupling work. All samples were dissolved in D_2O and the chemical shifts were recorded as δ (in parts per million) relative to an external tetramethylsilane signal at δ 0. Mass spectra were obtained with a Nuclide 12-90-G mass spectrometer at an ionization voltage of 70 eV and samples were introduced into the source with a direct insertion probe. Infrared spectra (KBr disks) were recorded with a Beckman IR-10 spectrophotometer. Ultraviolet spectra were obtained with a Unicam SP800 recording spectrophotometer.

Thin-layer chromatography was on Eastman Chromagram 6064 sheets (cellulose without fluorescent indicator) with the following solvent systems: A, 1% (v/v) NH_4OH; B, isopropyl alcohol–HCl–water (65:17:18, v/v); C, isopropyl alcohol–NH_4OH–water (70:10:20, v/v), D, *tert*-butyl alcohol–methyl ethyl ketone–HCl–water (40:30:10:20, v/v).

Phage and Bacterial Strains. Phage ϕW-14 was grown on *Pseudomonas acidovorans* 29 (Kropinski and Warren, 1970).

Preparation of Phage. CAA medium, rather than MB (Kropinski and Warren, 1970), was used for the preparation of phage in fermenters. CAA-M contained, in grams per liter: Casamino Acids, 12.5; yeast extract, 2.0; mannitol, 5.0; tryptophan, 0.05. Dow Corning C was used to suppress foaming. The cells were grown at 30° with high aeration (2 volumes of air/volume of medium min^{-1}). Phage was added when the culture reached an OD_{650nm} of 1.5.

Preparation of Phage DNA for Analysis. A phage lysate was centrifuged at 20,000*g* for 5 min to remove cellular debris. The turbid supernatant was decanted and centrifuged at 28,000*g* for 4 hr. The clear supernatant, containing less than 2% of the total plaque-forming units, was removed by suction and discarded. The phage pellet was allowed to resuspend for several days at 4° in 0.05 M Tris-HCl–0.01 M trisodium citrate–0.005 M NaCl buffer (pH 8.1). The highly turbid suspension was digested with DNase and RNase (each at 10 μg/ml) for 1 hr at 37°. Then the suspension was chilled to 4° and ice-cold 95% ethanol was added to a final concentration of 16%. After 2 hr the suspension was centrifuged at 6000*g* for 10 min, and the supernatant was removed and stored at 4°. DNA was released from the phage particles with 2.3 M sodium perchlorate (Freifelder, 1966); it was purified by the chloroform–isoamyl alcohol method (Marmur, 1961). DNA concentrations were estimated using an extinction coefficient of 20 cm^2/mg at 260 nm (Lee and Boezi, 1966).

Spectrophotometric Titration of DNA. ϕW-14 DNA dissolved in 0.5 M NaCl was titrated with freshly prepared 0.1 M NaOH in 0.5 M NaCl. The E_{260nm} was recorded at intervals and adjusted for the volume change during titration.

Spectral Properties of the DNA at pH 3. Samples of ϕW-14 DNA were dialyzed against 0.0015 M NaCl, then diluted 1:20 in 0.05 M acetic acid to give solutions of pH 3.0–3.1. Ultraviolet spectra were recorded at room temperature and used

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to calculate the ratio $E_{260nm}:E_{280nm}$. The mol % adenine plus thymine was obtained from a standard curve relating $E_{260nm}:E_{280nm}$ to mol % A + T (Fredericq *et al.*, 1961).

Molar Extinction Coefficient of DNA. The molar extinction coefficient, E_{260nm}(P) (Chargaff and Zamenhof, 1948), of ϕW-14 DNA was determined by assaying for inorganic phosphate (Chen *et al.*, 1956) in HCl hydrolysates of the DNA.

Melting Temperature Determinations. DNA melting temperatures (Mandel and Marmur, 1968) were determined in a Gilford 2400 automatic recording spectrophotometer fitted with a Model 2417 thermosensor (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The rate of heating was controlled automatically with a variable speed motor and controller. DNA samples were dialyzed against 0.0015 M NaCl prior to dilution in standard saline–citrate (SSC) of the appropriate concentration. *Escherichia coli* DNA was used as a standard.

Renaturation following thermal denaturation was examined using sheared DNA (Seidler and Mandel, 1971).

Buoyant Density Determinations. The buoyant densities of ϕW-14 DNA and of *P. acidovorans* DNA were determined under several conditions: neutral CsCl gradients (Mandel *et al.*, 1968); alkaline CsCl gradients, made up in 0.1 M Na_3PO_4 (Vinograd *et al.*, 1963); neutral Cs_2SO_4 gradients (Szybalski, 1968). *E. coli* DNA was used as a density standard.

Chemical Determination of Base Composition. Samples (1.0 mg) of the DNA were hydrolyzed with 90% formic acid. The bases in the hydrolysates were identified tentatively by descending chromatography on Whatman No. 1 paper, then separated by descending chromatography on Whatman No. 40 paper with isopropyl alcohol–HCl–water (65:17:18, v/v). Ultraviolet-absorbing areas were cut from the chromatogram and shaken with 5-ml amounts of 0.1 M HCl. Blanks were prepared from control areas of the chromatogram. The spectral characteristics of the solutions were determined and the bases were quantitated using their molar extinction coefficients.

Preparation of DNA from Large-Volume Lysates. After lysis was complete, cells and cellular debris were removed by passage of the lysate at 1 l./min through a cooled Sharples centrifuge. The supernatant was chilled to 4° and the phage was precipitated with 7% (w/v) Polyethylene Glycol 6000 (Yamamoto *et al.*, 1970). The suspension was left at 4° until the fine precipitate had settled out. The supernatant was siphoned off and discarded and the precipitate was collected by low-speed centrifugation. The crude phage pellet was resuspended in 0.05 M Tris-HCl–0.1 M trisodium citrate–0.005 M NaCl (pH 8.1) (phage buffer) and centrifuged at 6000*g* for 10 min. The supernatant was collected and centrifuged at 28,000*g* for 90 min, and the phage pellet was resuspended in phage buffer. Sodium dodecyl sulfate was added to the phage preparation (approximately 10^{16} pfu) to a final concentration of 2% (w/v). The suspension was heated to 45° for 15 min to ensure complete lysis. Phenol or chloroform isoamyl alcohol deproteinization proved difficult because of the large volume and high viscosity of the lysate, so Pronase digestion (50 μg/ml, 3 hr, 37°) was used to deproteinize the preparation. The crude DNA was precipitated with several volumes of ethanol, redissolved in 0.1 × SSC, and used without further purification.

Purification of the Unusual Pyrimidine. The DNA was precipitated from solution with 95% ethanol. The fibrous precipitate was washed with acetone and air-dried. Quantities (1 g) of the dried material were put into hydrolysis vials and dissolved in 16 ml of ice-cold HCl. After bubbling nitrogen through the solutions for 10 min, the DNA was hydrolyzed under reduced pressure at 100° for 90 min. The hydrolysates were filtered through Whatman No. 1 paper to remove precipitated material which interfered with subsequent purification steps. The filters were washed with 1 M HCl and the combined filtrates and washing were evaporated to dryness. Twice water was added to the residue and the solution was taken to dryness. The final residue was dissolved in a small volume of distilled water and the pH of the solution was adjusted to 6.8 with 0.01 M NH_4OH. The neutralized hydrolysate was applied to a column of CM-50 Sephadex (2.5 × 100 cm; NH_4^+ form). The column was washed with 0.005 M NH_4OH. Then 0.1 M HCl was passed through the column and the eluate was monitored for 260-nm absorption. The appropriate fractions were pooled and taken to dryness. The residue was dissolved in a small volume of 0.01 M HCl and applied to a column of Sephadex G-10 (2.5 × 100 cm), preequilibrated with this acid. The column was developed with 0.01 M HCl at a flow rate of 0.5 ml/min, and 3-ml fractions were collected. Fractions containing the unusual base were pooled and evaporated to dryness. The residue obtained was slightly yellow, but several triturations with small volumes of ice-cold absoute methanol left a colorless product which was collected, dried, and weighed. Approximately 160 mg of the pyrimidine was obtained from about 4 g of crude phage DNA. Only one ultraviolet-absorbing compound was detectable in the preparation by paper chromatography with several solvent systems, and ninhydrin–cadmium acetate reacted only with the ultraviolet-absorbing area of each chromatogram. The preparation was stored over P_2O_5. A small amount was recrystallized from methanol–1 M HCl for chemical analysis.

When desired, the product was converted to the free base by passing a solution of the dihydrochloride salt through a column of Dowex 50 (NH_4^+) and eluting the free base with 2 M NH_4OH.

Synthesis of N-Thyminylputrescine. 5-Bromomethyluracil[1] was synthesized from 5-hydroxymethyluracil (5-HmUra) by the method of Carbon (1960) with the following modifications. After the reaction was complete, the solution was cooled to room temperature and six volumes of anhydrous ether was added. The precipitate was allowed to settle out in the cold. The supernatant was discarded and the precipitate was washed four times with four volumes of ether. The finely granular product (97% yield) was dried *in vacuo* over KOH pellets. The infrared spectrum (ν_{max} 1670 and 1740 cm^{-1} (carbonyl stretching C(=O)NH) was devoid of absorption bands associated with hydroxyl groups.

The BrmUra (1.4 g, 6.8 mmol) was stirred slowly into putrescine free base (3 ml, 30 mmol). The mixture was kept at room temperature for 30 min, then acidified with concentrated HCl (5 ml, 60 mmol). Most of the precipitate was dissolved by the addition of a little water; insoluble material, assumed to be 5-HmUra and/or disubstituted putrescine, was removed by centrifugation. The supernatant was applied to a column of Sephadex G-10. Elution was with 0.01 M HCl; fractions of 3 ml were collected and those containing the monothyminylated product were pooled and evaporated to dryness. The residue was dissolved in HCl (0.01 M), applied to a Bio-Gel P-2 column, and the elution–concentration cycle was repeated until the product was free of putrescine.

[1] Abbreviations used are: 5-BrmUra, 5-bromomethyluracil; 5-HmUra, 5-hydroxymethyluracil; 5-HmCyt, 5-hydroxymethylcytosine.

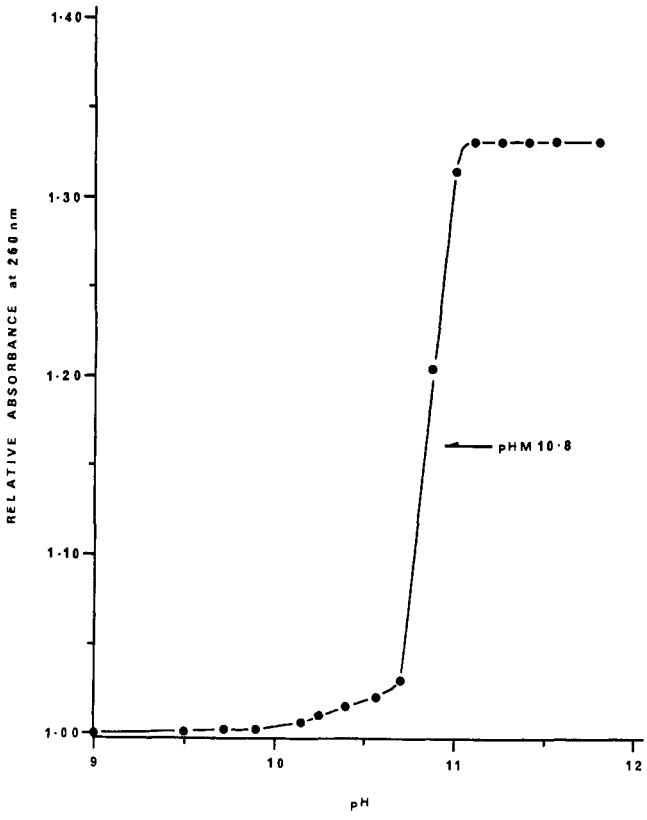


FIGURE 1: Spectrophotometric titration of φW-14 DNA.

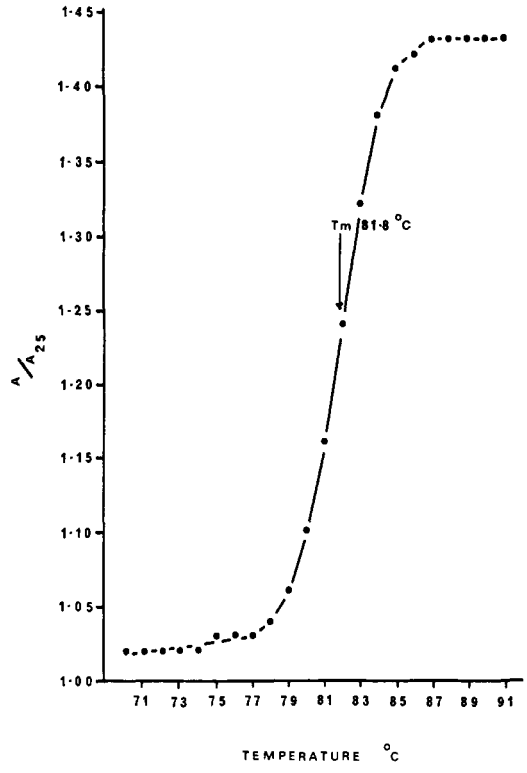


FIGURE 2: Thermal denaturation curve of φW-14 DNA in 0.042 X SSC. The melting temperature is indicated at the arrow.

Results

Spectral Properties of φW-14 DNA. In 0.1 × SSC: λ_{max} 258 nm, λ_{min} 263 nm; $E_{230nm}:E_{260nm}$ 0.425; $E_{280nm}:E_{260nm}$ 0.530. The values usually given by DNA for these ratios are 0.450 and 0.515, respectively (Marmur, 1963); spectrophotometric titration (Figure 1): pH M 10.85, hyperchromicity 30%. The pH M usually observed for strand separation is 11.7 (Dore and Frontali, 1968). The molar extinction coefficient is 6800. This is within the range for double-stranded DNA (Biswal *et al.*, 1967). $E_{260nm}:E_{280nm}$ (at pH 3.0) = 1.210. This corresponds to 40.5 mol % adenine plus thymine (Fredericq *et al.*, 1961).

Melting Temperature of φW-14 DNA. The melting curve of φW-14 DNA (Figure 2) was typical of double-stranded DNA (see Mandel and Marmur, 1968). The melting temperature in SSC was 99.75°. The thermally induced hyperchromicity was approximately 40% . Under similar conditions, the melting temperature of *E. coli* DNA was 92.30° (lit. (Mandel *et al.*, 1970) 91.85°). The melting temperature of φW-14 DNA was taken as 99.30° to compensate for the difference between the literature and recorded values for *E. coli* DNA.

The mol % guanine plus cytosine in φW-14 DNA was calculated using eq 1 (Mandel *et al.*, 1970).

$$\text{mol \% G + C} = 100\left[\frac{(T_m - 16.3 \log \text{rel SSC concn})}{50.2} - 0.990\right] \quad (1)$$

The value obtained was 66.3 mol % guanine plus cytosine. However, a more detailed examination of the thermal denaturation of φW-14 DNA showed that its melting temperature was not affected to the usual extent by the ionic concentration of the solvent. A tenfold change in the SSC concentration usually changes the melting temperature of DNA by 16.3° (Mandel *et al.*, 1970); the melting temperature of φW-14 DNA changed by only 13° (Figure 3). Substituting 13.0 for 16.3 in eq 1, the mol % guanine plus cytosine in φW-14 DNA was calculated to be 72.9.

The kinetics of renaturation of φW-14 DNA were quite similar to those of T even phage DNAs (Wetmur and Davidson, 1968). The second-order rate constants were 27 l. mol^{-1} sec^{-1} in SSC and 8 l. mol^{-1} sec^{-1} in 0.5 × SSC.

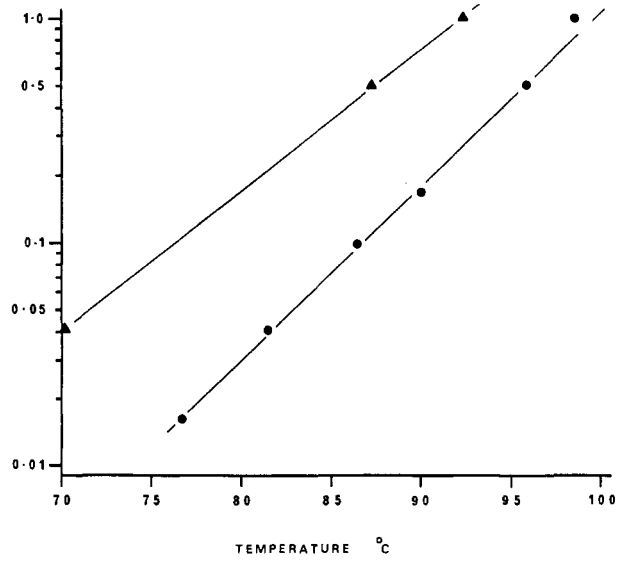


FIGURE 3: The variation of melting temperature with SSC concentration for *E. coli* DNA (▲) and φW-14 DNA (●).

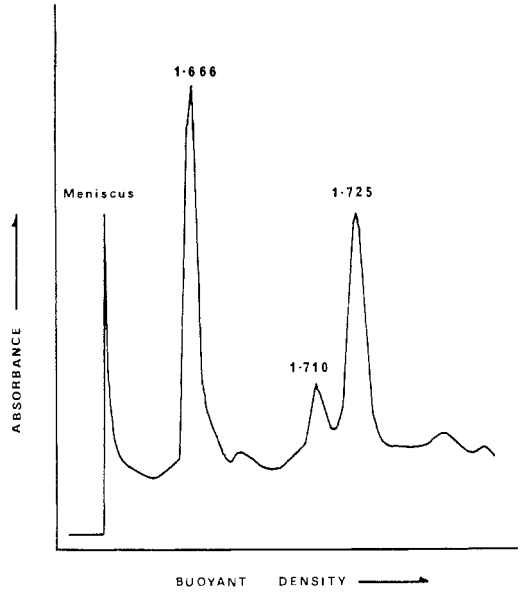


FIGURE 4: Microdensitometer tracing of ultraviolet absorption photograph of various DNAs in CsCl.Centrifugation for 20 hr at 20°. Buoyant densities: 1.666, ϕW-14; 1.710, *E. coli*; 1.725, *P. acidovorans*.

Buoyant Density of ϕW-14 DNA. The buoyant density of ϕW-14 DNA in neutral CsCl, assuming a value of 1.710 g/cm^3 for *E. coli* DNA, was 1.666 ± 0.0015 g/cm^3 (Figure 4). The buoyant density of *P. acidovorans* DNA was 1.725 g/cm^3 (Figure 4), which agreed with the reported value of 1.7255 g/cm^3 (Mandel, 1966). The buoyant density of ϕW-14 DNA was unaffected by the method of isolation or by treatment of the DNA with trypsin, pepsin, or cold acid followed by neutralization. The mol % guanine plus cytosine in ϕW-14 DNA was calculated using (De Ley, 1970): mol % G + C = 1020.6(ρ − 1.6606), where ρ is the buoyant density of the DNA. The value obtained was 4.5 mol % guanine plus cytosine.

Denaturation of the DNA, either with alkali followed by neutralization (Biswal *et al.*, 1967) or by heating followed by rapid chilling, increased its buoyant density by 0.014 g/cm^3.

The buoyant density of ϕW-14 DNA in Cs_2SO_4, assuming a value of 1.426 g/cm^3 for *E. coli* DNA, was 1.415 g/cm^3.

The buoyant density of ϕW-14 DNA in alkaline CsCl, assuming a value of 1.772 g/cm^3 for *E. coli* DNA under these conditions, was 1.750 g/cm^3.

TABLE I: Base Composition of ϕW-14 DNA Determined by Chemical Analysis.

Base	Mol %
Adenine	22.1 ± 0.4
Thymine	10.3 ± 0.2
Guanine	27.8 ± 0.2
Cytosine	28.4 ± 0.8
N-Thyminylputrescine[a]	11.9 ± 0.5
mol % G + C = 56.2	

[a] Determined by difference, assuming purine = pyrimidine, *i.e.*, thymine + cytosine + *N*-thyminylputrescine = adenine + guanine.

TABLE II: Chromatographic Properties of Nucleic Acid Bases.[a]

Base	R_F in Solvent System A	B	C	D
Adenine	0.53	0.33	0.63	0.32
Guanine	0.61	0.21	0.38	0.22
Cytosine	0.77	0.47	0.60	0.43
Thymine	0.87	0.80	0.77	0.86
N-Thyminylputrescine (DNA)	0.90	0.15	0.53	0.10
5-Aminouracil	0.90	0.22	0.49	0.23
Uracil	0.90	0.69	0.63	0.73
5-Hydroxymethyluracil	0.93	0.62	0.61	0.68
5-Carboxyuracil	0.96	0.63	0.24	0.75
5-Carboxymethyluracil	0.97	0.70	0.40	0.76
5-Hydroxymethylcytosine	0.82	0.46	0.55	0.43
N-Thyminylputrescine (synthetic)	0.89	0.15	0.53	0.10

[a] Sheets of Eastman Chromagram (6064), cellulose without fluorescent indicator. For solvent systems, see Analytical Methods.

The Actual Base Composition of ϕW-14 DNA. Hydrolysis of ϕW-14 DNA with formic acid (90%, 175°, 45 min) followed by paper chromatographic separation of the bases revealed the presence of five ultraviolet-absorbing compounds. Four of the compounds were identified by paper chromatography and their spectral characteristics in 0.1 M HCl and 0.1 M NaOH as adenine, thymine, guanine, and cytosine. The mol % guanine equalled the mol % cytosine, but there was almost twice as much adenine as thymine (Table I). Determination of the total extinction of thymine and the unusual base in various hydrolysates showed that the ratio of unknown:thymine varied from 0.4 with formic acid to 0.8 for HCl hydrolysis (6 M, 100°, 4 hr). Perchloric acid hydrolysis (70%, 100°, 60 min) destroyed the base completely. Hydrolysis with HCl resulted in some degradation of adenine.

Assuming that total purine equalled total pyrimidine, the mol % guanine plus cytosine in ϕW-14 DNA was 56.2 (Table I).

Preliminary Characterization of the Unusual Base. Comparison of the base with known bases by thin-layer chromatography on cellulose indicated that it was positively charged in acidic, but neutral in basic solvent systems (Table II). The formation of a pink color with ninhydrin–cadmium–acetate suggested that it was a primary amine. It did not correspond to any of the known pyrimidine bases.

Preparation and Purification of the Unknown Base. A mononucleotide derivative of the unusual base was not released during digestion of ϕW-14 DNA with pancreatic DNase and snake venom phosphodiesterase. Therefore, the free base was purified from HCl hydrolysates of the DNA. Attempts to purify the base by paper chromatography were frustrated by colored degradation products of the deoxyribose, which tended to obscure the ultraviolet-absorbing areas on the chromatograms, and by guanine, which tended to streak and thereby contaminate the unusual base. However, the desired compound was more basic than any other component in the hydrolysates, and was separated from most of them by chromatography on CM-Sephadex (Figure 5). The fraction rich in the unusual base was separated into two components by passage through a Sephadex G-10 column (Figure 6); the first

TABLE III: Typical Purification Scheme of the Unusual Pyrimidine from HCl Hydrolysate of ϕW-14 DNA.

Fraction	Total Vol. (ml)	Total OD_{260} Units	λ_{max}[a]	λ_{min}	Absorbance Ratios 250:260	280:260
Neutralized hydrolysate	2000	28,000	267	234	0.813	0.738
Hydrolysate after passage through CM-Sephadex	2034	21,560	267	234	0.821	0.762
NH_4OH (0.005 M) wash from CM-Sephadex	870	5,310	267	234	0.789	0.771
HCl (0.1 M) wash from CM-Sephadex	58	1,880	262	230	0.751	0.434
Peak 1 from Sephadex G-10			261[b]	229	0.753	0.280
Peak 2 from Sephadex G-10			282.5	241	0.65	2.16

[a] Spectral properties were determined after dilution with 0.1 M HCl. [b] The values for recrystallized *N*-thyminylputrescine are given in the text.

was the unknown base; the other was not identified but may have been a degradation product of adenine, which is labile during hydrolysis in HCl. The purification is summarized in Table III.

General Properties of the Unusual Base. The unusual base was white, crystalline, and highly water soluble; it decomposed at 255°.

When examined by thin-layer chromatography with a variety of solvent systems (Table II), the compound appeared to be more basic than any of the pyrimidines normally found in nucleic acids. In this respect it resembled 5-aminouracil, but the latter compound gave an orange color with the ninhydrin reagent, while the unknown base gave a pink color. *Anal.* Calcd for $C_9H_{18}Cl_2N_4O_2$: C, 37.82; H, 6.32; Cl, 24.91; N, 19.65; O, 11.23. Found: C, 38.08; H, 6.43; Cl, 24.57; N, 19.51; O, 11.41. Mol wt, 284.8. The presence of two chlorines suggested that the material was a dihydrochloride salt, in which case the empirical formula of the free base was $C_9H_{16}N_4O_2$, corresponding to a minimum molecular weight of 212.

Spectral Properties of the Unusual Base. The ultraviolet spectrum of the unusual base showed λ_{max} 261 (pH 1), 262 (pH 7), and 288.5 nm (pH 13); λ_{min} 229 (pH 1), 230.5 (pH 7), and 246 nm (pH 13); isobestic points 274 and 238.5 nm; E_{261} 7760 in 0.1 M HCl. The ultraviolet spectra were typical of those of 2,4-dihydroxypyrimidines. This was not unexpected, since the base replaced thymine in the phage DNA, and would have to hydrogen bond with adenine. The extinction coefficient of the unusual base was in the range found for other 2,4-dihydroxypyrimidines (*e.g.*, thymine, E_{264} 7900). The infrared spectrum of the unusual base was consistent with the presence of a 2,4-dihydroxypyrimidine; ν_{max} 3260 (N—H stretch of $-NH_3^+$) and 1740, 1685 cm^{-1} (carbonyl stretching C(=O)NH). The bands at 1650–1550 cm^{-1} were indicative of the N–H de-

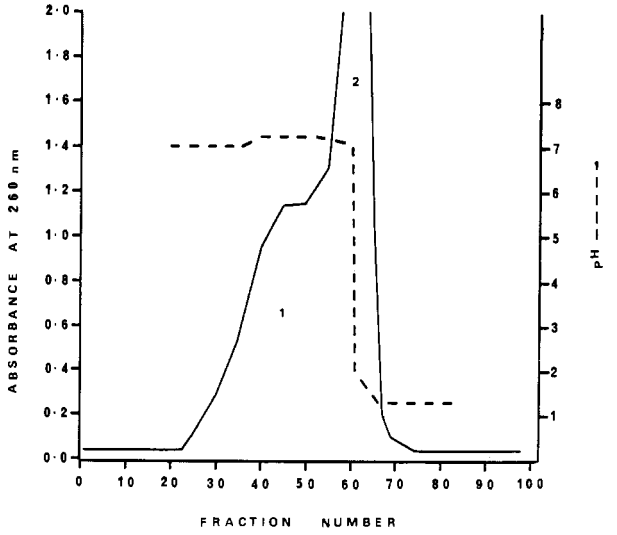


FIGURE 5: Elution of bases adsorbed to CM-Sephadex. The neutralized HCl hydroylsate of ϕW-14 DNA was passed through a column (2.5 × 100 cm) of CM-Sephadex, NH_4^+ form. The column was washed with several volumes of 0.005 M NH_4OH. Elution was with 0.1 M HCl; fractions of 3 ml were collected. Fraction 2 contained partially purified *N*-thyminylputrescine.

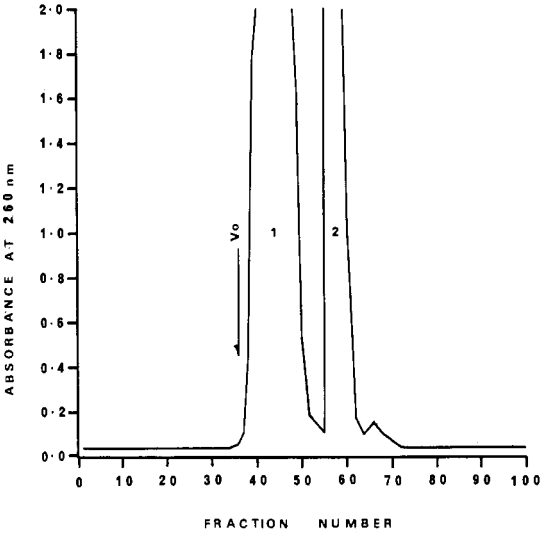


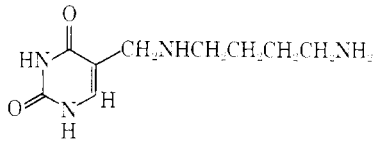
FIGURE 6: Purification of the unknown base on Sephadex G-10. The *N*-thyminylputrescine-containing fractions from the CM-Sephadex column were pooled and evaporated to dryness. The residue was dissolved in 0.01 M HCl and applied to a column (2.5 × 100 cm) of Sephadex G-10 in 0.01 M HCl. Elution was with 0.01 M HCl, at a flow rate of 0.5 ml min^{-1}. Fractions of 3 ml were collected. Peak 1 contained the *N*-thyminylputrescine. Peak 2 contained an unidentified uv-absorbing compound.

formations of primary and secondary protonated amine groups.

The 100-MHz nuclear magnetic resonance spectrum (D_2O) showed 8.25 (1 H, singlet), 4.47 (2 H, singlet), 3.56 (4 H, multiplet), and 2.25 (4 H, multiplet). The singlet at δ 8.25 was assigned by comparison with other pyrimidine spectra (Bhacca *et al.*, 1963) to the H-6 proton. Therefore, the uracil ring was not substituted at C-6. The singlet at δ 4.47 was assigned tentatively on the basis of comparison with the spectral properties of 5-CmUra (Gray and Lane, 1968) to an uncoupled methylene group at C-5. Spin–spin decoupling was used to determine the arrangement of the protons in the remaining two multiplets. Saturation of the protons at δ 3.56 caused the multiplet at δ 2.25 to collapse into a singlet, indicative of an ethylene moiety. Irradiation of the multiplet at δ 2.25 resulted in the collapse of the multiplet at δ 3.56 into a pair of singlets (δ 3.54 and 3.62). This confirmed the suspicion that the δ 3.56 multiplet was in fact overlapping triplets. Therefore, a butylene group appeared to form part of the side chain, and the chemical analysis and the lack of splitting of the δ 4.47 signal suggested that the side chain was $-CH_2NHCH_2CH_2CH_2CH_2NH_2$, which contains a putrescine (1,4-diaminobutane) group. The nmr spectrum of putrescine dihydrochloride was strikingly similar to that part of the unknown spectrum which was assigned to the 1,4-diaminobutane group: (D_2O) 3.66 (4 H, overlapping triplet, $-CH_2ND_2$) and 2.36 (4 H, multiplet, $-CH_2CH_2-$).

Proposed Structure of the Unusual Base. The chemical and spectral analyses were consistent with the unusual pyrimidine being thymine substituted with putrescine. The systematic name for the compound is 5-(4-aminobutylaminomethyl)uracil ($C_9H_{16}N_4O_2$; mol wt 212).

The name *N*-thyminylglycine was already in the literature (Cline *et al.*, 1958); therefore the compound isolated from ϕW-14 DNA was given the trivial name *N*-thyminylputrescine.



Mass Spectral Analysis of N-Thyminylputrescine. A parent ion (M^+) was not detected in the mass spectrum of *N*-thyminylputrescine. This is relatively common with amines, for which the parent ions are unstable. The major ion produced was of *m/e* 30. The ion $H_2C{=}N^+H_2$ is a major product of the fragmentation of primary aliphatic amino groups.

Properties of Synthetic N-Thyminylputrescine. Anal. Calcd for $C_9H_{18}Cl_2N_4O_2$: C, 37.82; H, 6.32; Cl, 24.91; N, 19.65; O, 11.23. Found: C, 37.70; H, 6.42; Cl, 24.77; N, 19.42; O, 11.39.

The nuclear magnetic resonance spectrum [(D_2O) 8.25 (1 H, singlet, H-6), 4.47 (2 H, singlet, $-CCH_2ND-$), 3.56 (4 H, overlapping triplets, $-NDCH_2-$ and $-CH_2ND$), and 2.25 (4 H, multiplet, $-CH_2CH_2$)], the infrared spectrum [ν_{max} 3260 (N—H stretch of NH_3^-) and 1740, 1685 cm^{-1} (carbonyl stretching C(=O)] and the ultraviolet spectra [λ_{max} 261 (pH 1), 262 (pH 7), and 288.5 nm (pH 13); λ_{min} 230 (pH 1), 230.5 (pH 7), and 245.5 nm (pH 13); isosbestic points 274 and 238.5 nm] of the synthetic product were identical with those of the natural product. The synthetic and natural products were indistinguishable by thin-layer chromatography (Table II), and both reacted with ninhydrin. Thus the structure proposed for the unknown base was confirmed by chemical synthesis.

Is N-Thyminylputrescine an Artefact Formed During Hydrolysis of ϕW-14 DNA? Like *N*-thyminylputrescine, 5-HmUra is destroyed by perchloric acid hydrolysis. This raises the question of the possible formation of *N*-thyminylputrescine from 5-HmUra in the DNA and free putrescine during hydrolysis of the DNA. Several observations argue against this possibility. 5-HmUra has never been observed in hydrolysates of ϕW-14 DNA; *N*-thyminylputrescine is not formed when a mixture of putrescine and 5-HmUra is subjected to the hydrolytic conditions used to release the unusual base from ϕW-14 DNA; free putrescine, as determined by paper chromatography (Ames and Dubin, 1960), is absent from purified ϕW-14 DNA, but *N*-thyminylputrescine is released by hydrolysis of the DNA.

After hydrolysis with either formic acid or HCl, the ratio thymine:adenine was approximately 0.5, whereas the ratio *N*-thyminylputrescine:thymine varied by a factor of almost 2. Therefore, it was unlikely that *N*-thyminylputrescine was formed from thymine during hydrolysis and *vice versa*.

Discussion

The unusual pyrimidine isolated from HCl hydrolysates of ϕW-14 DNA is 5-(4-aminobutylaminomethyl)uracil. It is, therefore, a hypermodified pyrimidine (Hall, 1971). Free putrescine is found in the particles of many phages (Cohen, 1971), but this is the first report of its being covalently bonded to DNA. Although it is unlikely that the compound is formed during hydrolysis, elucidation of the actual state of the base in ϕW-14 DNA is dependent upon the isolation of an *N*-thyminylputrescine nucleotide from the DNA.

The properties of ϕW-14 DNA are summarized in Table IV. Denaturation increases the buoyant density in neutral CsCl by 0.014 g/cm^3, which is within the range expected for DNA (Vinograd *et al.*, 1963). Therefore the single strands also have a very low buoyant density. The denatured DNA is homogeneous in density, a further indication that *N*-thyminylputrescine occurs in both strands. The sharp melting profile

TABLE IV: Properties of ϕW-14 DNA.

Characteristic	Obsd Value	Value Expected for 56 mol % G + C	Indicated mol % G + C
$E_{260nm}:E_{280nm}$ at pH 3.0	1.210	1.225	59.5
Melting temperature in			
SSC	99.3	94.1	66.3 (72.9)[a]
0.1 × SSC	86.3	77.8	
Buoyant density (g/cm^3) in			
Neutral CsCl			
Native	1.666	1.716	4.5
Denatured	1.680	1.731	
Alkaline CsCl	1.750	1.778	
Neutral Cs_2SO_4	1.415	1.428	
Base composition, chemical analysis			56.2

[a] After correction for the abnormal effect of the SSC concentration on the melting temperature of ϕW-14 DNA.

suggests that the hypermodified base is fairly uniformly distributed throughout φW-14 DNA.

Besides its buoyant density and melting temperature, φW-14 DNA is also unusual in the level and type of replacement of a normal base with a modified one. Trace amounts of modified bases are common in many bacteriophage DNAs (Hall, 1971), and in some bacteriophage DNAs a pyrimidine is replaced completely with a modified pyrimidine (Mandel, 1968). However, φW-14 DNA is unusual in that (a) half the thymine is replaced, (b) the pyrimidine replacing thymine is hypermodified, and (c) the replacement lowers the buoyant density and raises the melting temperature of the DNA. The complete replacements observed previously usually increase the buoyant densities and lower the melting temperatures of the DNAs (see Mandel, 1968). However, φW-14 DNA may not be unique. In the DNA of *Serratia marcescens* phage η, guanine is partially replaced with a fifth base (Pons, 1967). The buoyant density of *Bacillus cereus* phage PR DNA is 1.659 g/cm^3, and the DNA is partially resistant to deoxyribonuclease (Levin and Compans, 1968). φW-14 DNA is partially resistant to pancreatic deoxyribonuclease and to snake venom phosphodiesterase. Recently, 5-(4,5-dihydroxypentyl)uracil has been shown to replace almost half the thymine residues in the DNA of *Bacillus subtilis* phage SP-15 (Krasuski *et al.*, 1972). However, the buoyant density of SP-15 DNA is higher and the melting temperature is lower than expected for the base composition of the DNA.

DNA is stabilized by free polyamines, the increased stability being reflected by a rise in melting temperature (Tabor, 1962; Mahler and Mehrotra, 1963). The effect of a given polyamine on the melting temperature decreases as the ionic strength of the solvent is increased (Tabor, 1962; Mahler and Mehrotra, 1963). φW-14 DNA behaves like a DNA–polyamine complex, the deviation of its melting temperature from the theoretical decreasing by 3.3° for each tenfold increase in the ionic strength of the solvent (Figure 3). Polyamines have very little effect on the ultraviolet spectral properties of DNA (Mahler and Mehrotra, 1963), and those of φW-14 DNA do not appear unusual. The spectral properties of *N*-thyminylputrescine are quite similar to those of other uracil-type pyrimidines.

The sedimentation rate of DNA is decreased markedly by polyamines (Mehrotra and Mahler, 1964). The buoyant density of phage PBS2 DNA, in which uracil substitutes for thymine (Takahashi and Marmur, 1963), is increased by the substitution. In other words, methylation of uracil decreases the buoyant density of the DNA. Alkylation of DNA with nitrogen mustard (*N*-methylbis(2-chloroethyl)amine·HCl), which results predominantly in the addition of $-CH_2CH_2^+NH(CH_3)CH_2CH_2OH$ groups to the N-7 positions of guanine residues, reduces the buoyant density of the DNA; the hypodensity decreases when the pH is raised above 9.5 (Kohn and Spears, 1967). The hypodensity of φW-14 DNA is decreased in alkaline CsCl.

Most of the unusual physical properties of φW-14 DNA appear to be a consequence of and consistent with the covalent bonding of putrescine to thymine. However, the presence of *N*-thyminylputrescine would be expected to raise rather than lower the pH M for the dissociation of the double helix, and to accelerate renaturation following thermal denaturation.

The role of *N*-thyminylputrescine in φW-14 DNA is unclear. It may, like glucosylated 5-HmCyt (Revel and Luria, 1970), be involved in host-controlled modification. Alternatively, it may aid in DNA packaging by neutralizing negative charges.

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