A NEW NITROGEN BASE 5-HYDROXYCYTOSINE IN PHAGE N-17 DNA

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1. Introduction

It is known that phage DNA may contain abnormal bases besides the usual ones, for example, 5-hydroxymethylcytosine and 5-methylcytosine in place of cytosine or 5-hydroxymethyluracil and 5-dihydroxypentyluracil instead of thymine [1]. In all the cases reported so far there are side substituents at the 5-position of the pyrimidine ring, which are linked to the heterocycle by a C–C bond. In contrast to that we have identified an essentially new type of abnormal base, which completely substitutes for cytosine in double-stranded DNA of N-17 dysentery phage. Hydroxy group of the new base is linked directly to the pyrimidine ring. Some results concerning the possible structure of this compound were reported [2].

2. Materials and methods

N-17 phage was grown in *Shigella flexneri*. Newcastle and purified as in [3] and DNA was extracted with phenol [4]. The DNA concentration was determined from the phosphorus content [5].

The DNA was hydrolysed to its constituent bases by 2 h heating at 100°C in 6 N HCl [6]. The nucleotide composition was determined by paper chromatography on Whatman no. 1 in methanol–conc. HCl–H_2O (7:2:1) mixture [7]. The preparative isolation of 5-hydroxycytosine was accomplished by Dowex 1 X 2 (OH^--form) chromatography with 0.1 M NH_4HCO_3, at pH 9.8. The additional purification of the base was performed by rechromatography on Dowex AG 50 X 2 (H^+-form) with 0.1 M NH_4OH.

DNA apurinization was accomplished either by incubation in 0.1 M HCl for 8 h at 37°C or by incubation with Amberlite CG-120 (H^+-form) for 10–14 days at 20°C [8]. In the first case opalescence of the solution disappeared in the course of incubation and its UV spectrum did not change further.

SD phage DNA, which contains the 4 usual bases, was used as a control throughout [9].

CsCl and Cs_2SO_4 density gradient centrifugation was done in a Spinco E (Beckman) centrifuge, AnG-Ti rotor, 44 000 rev./min, 25 ± 0.1°C.

The following instruments were used for spectroscopic measurements: Pye Unicam SP-8000 (UV-spectroscopy), Varian-100 (NMR), Roussel-Jouan CD-185 (CD) and MX-1303 mass-spectrometer (USSR).

3. Results and discussion

Chromatography of phage N-17 DNA HCl–hydrolyzate on Dowex 1 X 2 (fig.1) easily allows separation of the abnormal base, which is washed out with 0.1 M NH_4HCO_3 (pH 9.8) a little later than control cytosine. Thymine, guanine and adenine were retarded on the column and can be eluted with 1 M NH_4Cl. While elution pattern of the abnormal base showed its relationship to cytosine, it showed a small reduction in its basic properties. There were only trace quantities of cytosine in the N-17 DNA hydrolyzate which means that all cytosine in the DNA is replaced by this new derivative.

Mass spectrometry confirmed that the new base was an analogue of cytosine (fig.2). Its molecular ion was 127, that exceeds the cytosine mass by only 16. The characteristic spectral peaks indicate elimination of the NH_2 groups (*m/e* 111), CO (*m/e* 99), NCO (*m/e* 85) and HNCO (*m/e* 84). Thus its can be concluded that the new compound is dihydroxyaminopyrimidine.

The UV absorption spectrum of the purified new base exhibits the presence of an additional substituent which is highly conjugated with the pyrimidine ring

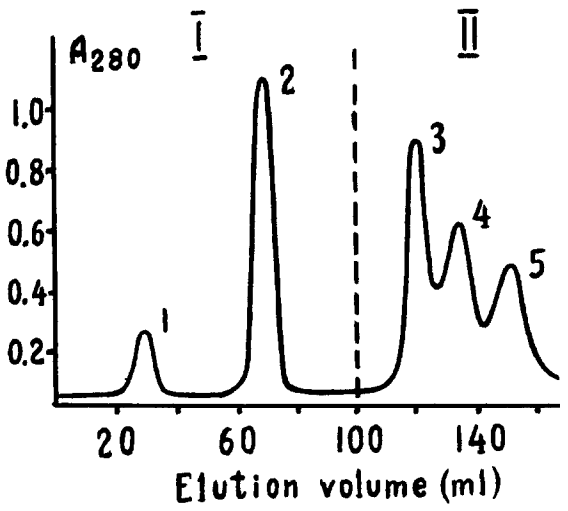


Fig.1. Chromatography of nitrogen bases on Dowex 1 × 2, 1.4 × 12 cm column, flow rate of 2 ml/min: (I) 0.1 M NH_4HCO_3 (pH 9.8); (II) 1 M NH_4Cl (pH 9.8); (1) cytosine (a marker); (2) abnormal base; (3) thymine; (4) guanine; (5) adenine

π-electrons (fig.3). While the cytosine absorption maxima in acidic and alkaline media are localized at 276 and 280 nm, for the abnormal base they are at 298 and 312 nm, respectively. Since the N-17 phage DNA is resistant to enzymatic hydrolysis (V. V. S., unpublished), we have failed to isolate the abnormal base in the nucleotide form and to express its absorption through ϵp. Molar extinction coefficients of the abnormal base were calculated from the nucleotide composition of N-17 phage DNA obtained from paper chromatography. The contents of adenine, thymine and guanine in the DNA were 0.315 ± 0.08, 0.329 ± 0.01 and 0.175 ± 0.01 mol/mol phosphorus. Since the Chargaff rule is obligatory for double helical DNA and A:T is ~1, the content of the abnormal base can be regarded as equal to the guanine content in N-17 DNA. The accuracy of this approach is confirmed by the data given below. The calculated molar extinction coefficients presented in table 1 are usual for pyrimidines.

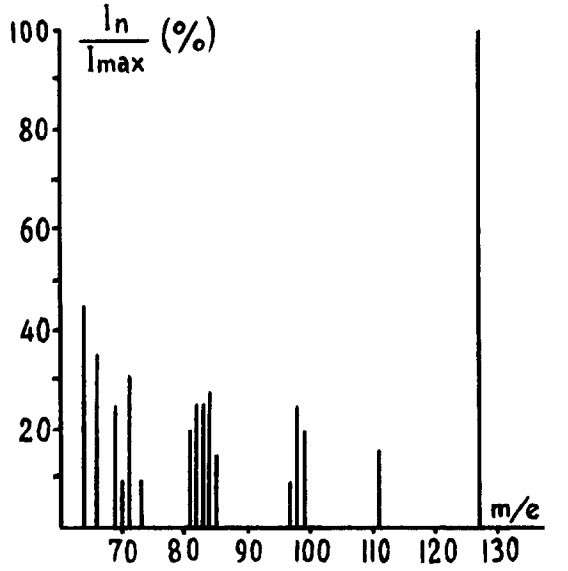


Fig.2. Mass-spectrum of the abnormal base. Ionization voltage 30 V, 290°C.

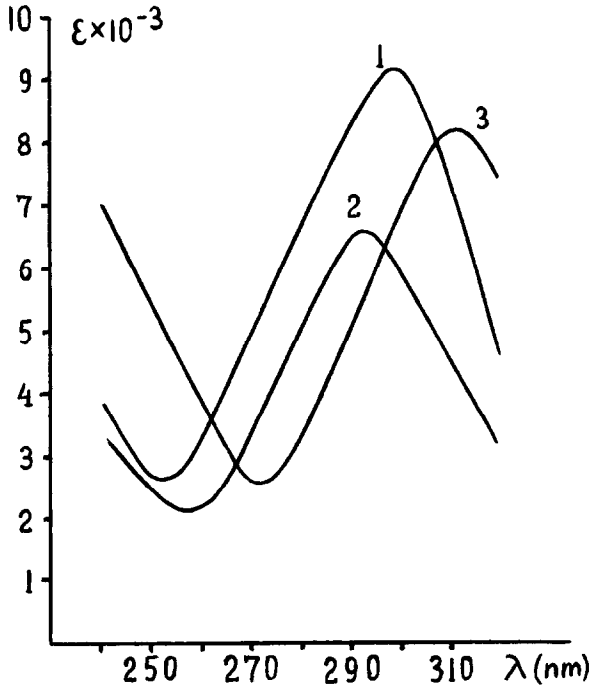


Fig.3. UV absorption spectra of the abnormal base: (1) 0.1 M HCl; (2) 0.1 M phosphate buffer (pH 7.0); (3) 0.1 M NaOH.

The nuclear magnetic resonance (100 Hz) spectrum of the new base in DMSO is lacking proton resonances at the 5 and 6 positions of the cytosine

Table 1
Spectral properties of 5-hydroxycytosine

Solvent	λ_{max}	ϵ
0.1 M HCl	298	9.1×10^3
Phosphate buffer (pH 7.0)	292	6.6×10^3
0.1 M NaOH	312	8.3×10^3

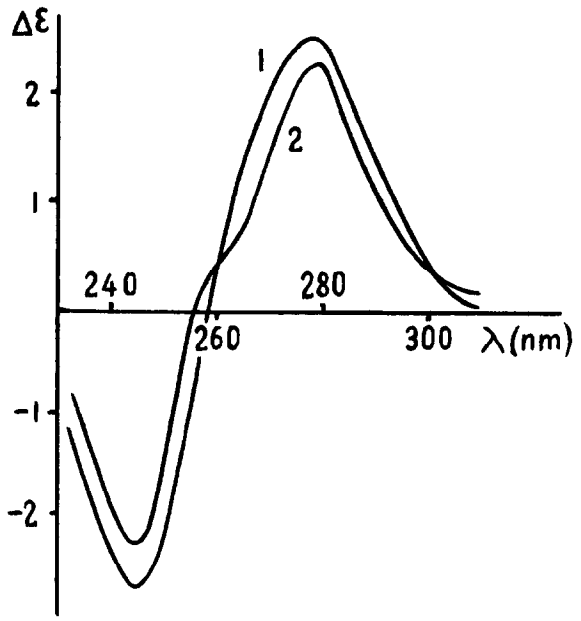
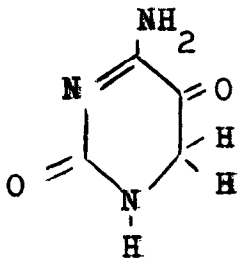


Fig.4. Circular dichroism spectra of DNA in 0.1 M NaCl: (1) SD phage DNA as a standard; (2) N-17 phage DNA.

ring (δ 5.57 and 7.31) but the broad methylene group resonance (δ 3.50) is apparent. Upon addition of D_2O to the solvent this resonance disappears due to the proton mobility, indicating ketoenol tautomerism.

Regarding the possible localization of the additional hydroxy group at either position 5 or 6 of the cytosine ring, one should keep in mind that the presence of a subsituent at the 6 position hinders the formation of the double helix in DNA [10]. At the same time, in the CD spectrum of the N-17 phage DNA the commensurable positive (278 nm) and negative (246 nm) bands and the intersection point (257 nm) (fig.4) indicate that the N-17 DNA has a usual B-conformation.

Thus, the data above allow us to consider the new base in N-17 phage DNA to be 2,5-dihydroxy-4-aminopyrimidine or 5-hydroxycytosine (5-HC):



Although the UV spectrum maximum of 5-HC is ~300 nm (fig.3) the spectrum of native N-17 DNA does not show a marked absorption in this region in neutral or alkaline media (fig.5). So we suppose that the 5-HC hydroxy group in the DNA is blocked and is released from this blocking only during DNA hydrolysis. To check this assumption, N-17 phage DNA was subjected to acidic treatment under the mild conditions of apurinization. The UV spectra of the native N-17 DNA and of the apurinization mixture are shown in fig.5. Incubation for 8 h of N-17 DNA with 0.1 M HCl at 37°C resulted in an increase of 1.65×10^3 in εp at 298 nm. The theoretical εp increase is 1.6×10^3 as estimated from the 5-HC content of N-17 DNA (~0.175 mol/mol phosphorus) and the molar extinction coefficient (9.1×10^3) of 5-HC in 0.1 M HCl. The close agreement between the directly obtained and calculated values indicates that either all or at least the major part of 5-HC in the

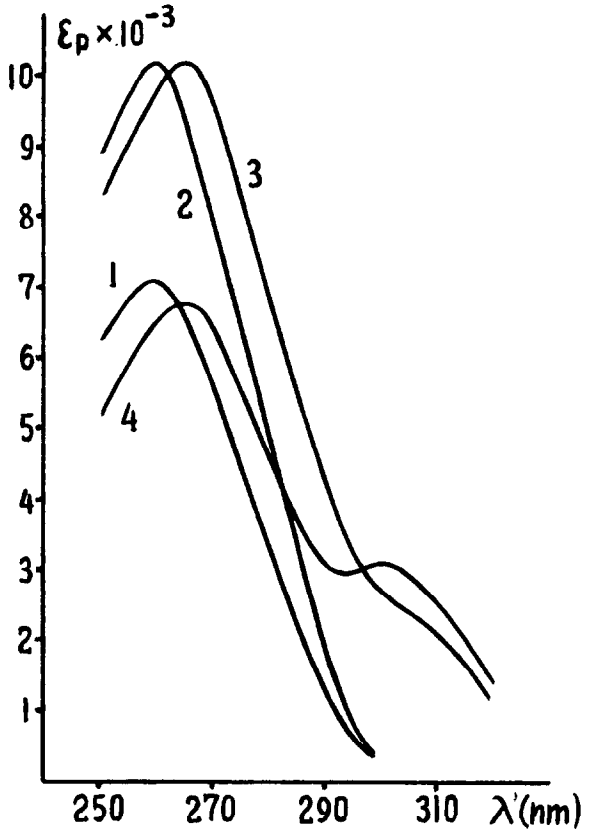


Fig.5. Apurinization of N-17 phage DNA: (1) native DNA in 0.1 M NaCl; (2) native DNA in 0.2 M NaOH; (3) DNA after 0.1 M HCl treatment; (4) apurinic acid after Amberlite CG-120 treatment.

Table 2
Buoyant density of N-17 DNA

	CsCl	Cs_2SO_4
ρ (g/cm^3)	1.709	1.447
G + C calc. (%)	49	90
G + C exp. (%)	35	

DNA is really blocked by some groups which are cleaved during apurinization. On apurinization of N-17 DNA with Amberlite CG-120, when purines remain on the ion exchanger, the 300 nm absorption increase is even more pronounced (fig.5), showing that this increase is due to the pyrimidine fraction of the DNA.

The SD phage DNA used as a control does not show any changes at this region of the UV spectrum upon the same treatment.

Concerning groups which block 5-HC in the native DNA the buoyant density of the DNA in solutions of cesium salts can serve as an indirect indication of its nature (table 2). It is well known that the DNA buoyant density (ρ) value depends on the (G + C)/(A + T) ratio and the main factor which affects this relationship is the additional glycozilation of DNA [11]. On the basis of the N-17 DNA nucleotide composition and ρ_{obs} in CsCl (1.709 g/cm^3) and Cs_2SO_4 (1.447 g/cm^3) one may assume that N-17 DNA contains an extra component of a carbohydrate nature.

The sugar origin of this component is also supported by the ease of its elimination during acidic treatment and by resistance of N-17 DNA to different phosphodiesterases as well as to restriction endonucleases (V. V. S., unpublished).

A similar base exists in a phage of *Shigella sonnei* [12]. The base structure has been proposed on the basis of the similarity of the UV spectrum of the new base and of synthetic 5-HC. However, it was concluded [12] that this base appears as a result of degradation of some other base of unknown structure during the hydrolysis in concentrated $HClO_4$. Besides, the extreme instability of this cytosine analogue was [12]. We must emphasize that the different mild treatment of N-17 DNA used in our experiments can split only comparatively weak chemical bonds like O- or N-glucosidic ones. Concerning the chemical stability of the free 5-HC our conditions of treatment and isolation of this compound did not produce any destruction. Thus, 5-HC with free or blocked hydroxy group should be a true constituent of N-17 phage DNA.

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