MECHANISM OF THE MUTAGENIC ACTION OF HYDROXYLAMINE

SYNTHESIS OF CYTIDINE DI- AND TRIPHOSPHATES MODIFIED BY HYDROXYLAMINE AND O-METHYLHYDROXYLAMINE

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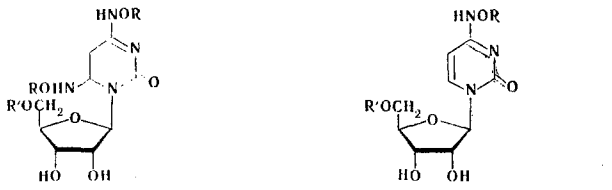
The reaction of hydroxylamine and O-methylhydroxylamine with cytidine 5'-di- and triphosphates gave nucleoside di- and triphosphates containing hydroxylamine and O-methylhydroxylamine residues in the heterocyclic ring. At low temperatures, high pH values, and high hydroxylamine concentrations, the chief products are 4,6-dihydroxylamino-5,6-dihydro-2(1H)-pyrimidinone derivatives, while 4-hydroxylamino-2(1H)-pyrimidinone derivatives are primarily formed when the temperature is raised and the pH and hydroxylamine concentration are lowered.

One of the chief reasons for the mutagenic action of hydroxylamine (III) and O-methylhydroxylamine (IV) is apparently modification of the cytosine ring by these agents [1, 2]. The validity of this assumption can be verified in vitro, particularly in the RNA-polymerase system, by studying the functional activity and specificity of the modified bases in the composition of the starting nucleoside triphosphates or in the composition of the matrix, for the production of which the corresponding nucleoside diphosphates are necessary.

It is known that two products – 1-β-D-ribofuranosyl-4,6-dihydroxylamino-5,6-dihydro-2-pyrimidinone (Ia) and 1-β-D-ribofuranosyl-4-hydroxylamino-2-pyrimidinone (IIa) – are forned in the reaction of cytidine with hydroxylamine [1-3]. Similar derivatives (Ic and IIc) are formed in the reaction of cytidine with O-methylhydroxylamine.

Compound IIe was previously synthesized by Janion and Shugar [4]. The synthesis of IIe and IIf from Ie and If, obtained by the reaction of III or IV with cytidine diphosphate, has also been described [5].

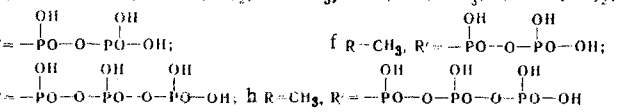
A study of the mechanism and the kinetics of the reaction of cytidine with III and IV demonstrated that the ratio (II/I) of the concentrations of II and I depends substantially on the reaction conditions [3]. This



I a-h

II a-h

I, II a R=H, R'=H; b R=H, R'=PO(OH)$_2$; c R=CH$_3$, R'=H; d R=CH$_3$, R'=PO(OH)$_2$;



e R=H, R'=; f R=CH$_3$, R'=;

g R=H, R'=; h R=CH$_3$, R'=

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ratio increases as the temperature is raised and the pH and reagent concentration are lowered. The conditions can be selected in such a manner that practically only one product will be formed in the reaction mixture. This circumstance was used for the synthesis of diphosphates IIe, f and triphosphates Ig, h, and IIg, h.

There is no absorption at 260-280 nm in the UV spectra of I, while II have characteristic spectra at 220-320 nm [6]. This makes it possible to identify the type of base in the products of the modification of the di- and triphosphates, since the presence of phosphate groupings as substituents in the carbohydrate residue has virtually no effect on the electronic spectra of the bases. The identification of the mono-, di-, and triphosphates is readily accomplished by utilizing the difference in their electrophoretic and chromatographic mobilities. Triphosphate Ig, which was practically free of the starting cytidine triphosphate, and IIg could be obtained after 6 h at room temperature and pH 6.5 by using high concentrations of III (6-7 M). This conclusion can be drawn on the basis of the UV spectrum of the reaction mixture, which does not have absorption at 250-320 nm.

Only a 5 M solution can be obtained from the hydrochloride of IV. In addition, when the concentrations and other modification conditions are identical, the II/I ratio in the case of IV is considerably higher than in the case of III [3]. Thus, when the modification is performed with 5 M IV at pH 6 and room temperature, the spectrum of the reaction mixture no longer changes after 6 h, and therefore the reaction has ceased, but appreciable absorption due to the formation of a certain amount of IIf is observed at 250-300 nm.

Polyphosphates IIe-IIh were obtained under standard conditions at low hydroxylamine concentrations (1 M), a high temperature (55-56°), and a rather low pH (5). It should be noted that in the case of the polyphosphates, a marked decrease in pH can lead to appreciable degradation of the polyphosphate grouping.

In the case of III, the spectrum of the reaction mixture no longer underwent change after 5-6 h, as compared with 6-7 h in the case of IV. Moreover, the spectra of the mixtures after removal of III or IV attested to the fact that products of the II type are primarily formed in the mixture.

In addition to modification of the heterocyclic base, degradation of the pyrophosphate grouping is also possible in the case of pyrophosphate derivatives. This process can be accelerated when the pH is reduced and the concentration of III and IV is raised. While purifying the products, we isolated the mono- and diphosphate derivatives and were able to estimate the degree of degradation of the pyrophosphate group under the modification conditions (Table 1). The degradation of the pyrophosphates is not very high, and the modification products can be obtained in good yields. The amount of residual undegraded triphosphate is the same both at high temperatures and low pH values (conditions that promote decomposition of the pyrophosphate) and at low temperatures and high pH values (conditions under which the triphosphates are stable). Consequently, an increase in the concentration of III in the second case leads to acceleration of the degradation of the pyrophosphate bond.

The purity of the products obtained is of especially important significance for the investigation of the functional activity. In addition to degradation of the pyrophosphate group, deamination of the cytosine ring to uridine and the presence of a certain amount of the starting compound are possible during the modification; in addition I may contain admixtures of products of the II type and vice versa. Consequently, one needs those conditions of purification under which it is possible to separate the reaction products that differ both in the structure of the heterocyclic ring and in the degree of phosphorylation.

We have previously [7] determined the pK_a values of monophosphates IIb and IId, which proved to be 2.26 and 1.18 respectively, which is substantially lower than the pK_a of cytidine monophosphate (4.5) [8]. It must be assumed that the pK_a values of products Ib and Id have large values if the same regularity in the change in the pK_a is observed as on passing from cytidine to dihydrocytidine [8]. One might therefore hope to separate the products by using the difference in the charges of their bases in the acid pH region. Since degradation of the pyrophosphate group is possible at such low pH values, pH 5 was selected for the separation inasmuch as the cytosine ring is protonated to a considerable extent at this value, while the ring in II is virtually uncharged. The separation was carried out on AG 1×8 anion-exchange resin. Standardized ion-exchange chromatography was used to identify the mono-, di-, and triphosphates of cytidine and uridine and monophosphates Ib, d and IIb, d. It is apparent from Table 2 that triphosphates IIg, h can be reliably separated from all of the possible impurities under the conditions used. Under these conditions, triphosphates Ig, h are separated from uridine triphosphate and from triphosphates IIg, h, but they may contain diphosphates IIe, f and cytidine triphosphate. In order to avoid admixtures of IIe, f, an additional step – prior separation with respect to the charges of the phosphate groups, which was carried out by ion-exchange chromatography on DEAE-Sephadex at pH 8.6 – was introduced for the purification of Ig, h. Under these conditions,

TABLE 1. Percent Yields of Mono-, Di-, and Triphosphates in the Modification of Cytidine Triphosphate with Hydroxylamine and O-Methylhydroxylamine

Type of nucleotide isolated from the reaction mixture	Modifying agent and conditions			
	III		IV	
	6–7 *M*; pH 6,5, 20°, 6 h	0,85 *M*; pH 5, 54°, 6 h	5 *M*; pH 6, 34°, 5 h	0,85 M; pH 5, 54°, 7 h
Monophosphate	7	13	7	17
Diphosphate	22	12	25	8
Triphosphate	71	75	68	74

TABLE 2. Sodium Chloride Concentrations (mole/liter) That Cause Elution of the Investigated Compounds*

Heterocyclic ring	Monophosphate	Diphosphate	Triphosphate
I†	0,06±0,01	0,14±0,01	0,24±0,1
II†	0,15±0,01	0,23±0,01	0,34±0,01
Cytosine	0,05±0,01	0,12±0,01	0,21±0,01
Uracil	—	0,14±0,02	0,27±0,01

*Ion-exchange resin AG 1×8 (200-400 mesh), 1 by 8 cm column, eluting solution 0.005 M acetate buffer with pH 5, NaCl linear gradient 0→0.4 M, total volume of eluate 500 ml.

† The characteristics of the hydroxylamine and O-methylhydroxylamine derivatives were identical.

TABLE 3. Triethylammonium Bicarbonate Concentrations (mole/liter) That Cause Elution of the Indicated Compounds*

Heterocyclic base	Monophosphate	Diphosphate	Triphosphate
I	0,14±0,01	0,21±0,02	0,29±0,02
II	0,16±0,01	0,24±0,01	0,34±0,01
Cytosine	0,12±0,01	0,19±0,01	0,29±0,02
Uracil	0,15±0,01	0,23±0,01	0,32±0,02

*Ion-exchange resin DEAE-Sephadex A-25 (HCO_3^- form), column 2×20 cm, triethylammonium bicarbonate linear gradient at pH 8.6, 0.1→0.5 M, total volume of eluate 2000 ml.

TABLE 4. Spectral Characteristics of Triphosphates Obtained As a Result of Modification of Cytidine Triphosphate with Hydroxylamine and O-Methylhydroxylamine

Triphosphate	pH	λ_{max}, nm	E_{max}/E_{270}
Ig	7	225	—
Ih	7	230	—
IIg	7	235, 270	1,9±0,1
IIh	7	242, 272	1,6±0,1

partial separation of products I and II (Table 3) was also observed. After separation in this system, the fractions corresponding to the triphosphates were desalinized and chromatographed in the first system to give triphosphates Ig, h, which were free of diphosphates and uridine triphosphate and triphosphates IIg, h. The spectral characteristics of the products obtained in this manner (Table 4) are analogous to the characteristics of the corresponding monophosphates [6]. Thus the triphosphates (Ig, h and IIg, h) that we obtained are free of the possible impurities.

EXPERIMENTAL

The spectroscopic determinations were made with an SF-4A spectrometer. The extinction of the products was determined starting from the phosphorus concentrations in solutions of the compounds with known optical density. The phosphorus concentration was determined by the method in [9] after decomposition of the compounds with concentrated sulfuric acid. The molar concentrations of the cytidine phosphates were determined by spectrophotometry in neutral solutions using a molar extinction (ε) of $7.4 \cdot 10^3$ [10]. Electrophoresis on FN-12 paper was carried out with 0.05 M citrate buffer with pH 5 with a voltage gradient of 7.5 V/cm for 3-4 h. The mobilities of the di- and triphosphates relative to the monophosphates under these conditions were 1.3 and 1.5, respectively.

Cytidine Triphosphate. The preparation produced by the Reanal Company was purified by ion-exchange chromatography by the following method. A 100-200 mg sample of the cytidine triphosphate in 3-5 ml of water was placed on a column filled with DEAE-Sephadex (20 × 2 cm) in the bicarbonate form. The column was equilibrated with 0.1 M triethylammonium bicarbonate buffer (pH 8.6, linear gradient 0.1 → 0.5 M, eluate volume 2000 ml). The fraction containing the triphosphate (Table 3) was evaporated, and the residue was dissolved in water. A 5-7 ml sample of KM-Sephadex was added to the aqueous solution, and the mixture was stirred for 20 min. The Sephadex was removed by filtration, and the solution was neutralized with alkali and lyophilized.

Hydroxylamine Solution. Analytical grade hydroxylamine hydrochloride was purified by recrystallization from water. To prepare an 8 M solution of the free base, a concentrated solution of potassium hydroxide was added with cooling and vigorous stirring to a suspension of the calculated amount of a suspension of the hydrochloride in water. The pH was then brought up to the assigned value, and the precipitated potassium chloride was removed by filtration. The solution was used for the reactions.

O-Methylhydroxylamine Solution. O-Methylhydroxylamine was prepared by the method in [11] and purified by distillation of the free base obtained by neutralization with concentrated alkali solution to give a product with bp 49-50°. To obtain solutions of the needed concentration, the calculated amount of the free base was dissolved in water, the solution was cooled, and the necessary pH was obtained by addition of hydrochloric acid.

1-β-D-Ribofuranosyl-4,6-dihydroxylamino-5,6-dihydro-2-pyrimidinone 5'-Triphosphate (Ig). A total of 2.5 ml of 8 M hydroxylamine solution with pH 6.5 was added to 0.5 ml of 0.2 M cytidine triphosphate, and the mixture was incubated at 20° for 6 h. The hydroxylamine was separated by gel filtration in a column (5 × 50 cm) filled with Sephadex G-10 using water as the eluting agent. The fraction containing a mixture of phosphates was chromatographed as described in the footnotes to Tables 2 and 3. Desalinization after chromatography in the triethylammonium carbonate system was accomplished by evaporation in a film evaporator and subsequent treatment with KM-Sephadex to remove traces of triethylamine (see above). Desalinization after chromatography in the acetate buffer—NaCl system was realized either by gel filtration through Sephadex G-10 or by dilution of the corresponding fraction to an NaCl concentration of 0.01 M and passage of the dilute solution through a 1 × 50 column filled with DEAE-Sephadex in the bicarbonate form. The Cl^- ions were washed out with 0.01 M bicarbonate buffer, the adsorbed nucleotides were eluted with 0.5 M triethylammonium bicarbonate (pH 8.6), and the triethylamine was removed as described above. The preparations were lyophilized after desalinization and stored in the dry form in a refrigerator. The yield of Ig was 35-40% of the starting cytidine triphosphate.

1-β-D-Ribofuranosyl-4,6-di(O-methylhydroxylamino)-5,6-dihydro-2-pyrimidinone 5'-Triphosphate (IIh). A total of 2.5 ml of 5 M IV with pH 6 was added to 0.2 ml of 1 M cytidine triphosphate, and the mixture was incubated at 34° for 5 h. Workup of the mixture was carried out as in the preparation of Ie to give 35-40% IIh.

1-β-D-Ribofuranosyl-4-hydroxylamino-2-pyrimidinone 5'-Di- and Triphosphates (IIe, g). A total of 2.5 ml of 1 M III with pH 5 was added to 0.5 ml of 0.2 M cytidine di- or triphosphate, and the mixture was incubated at 54° for 6 h. It was then worked up as in the preparation of Ie, g to give 25-30% (based on the starting cytosine derivatives) of pure IIe and IIg.

1-β-D-Ribofuranosyl-4-(O-methylhydroxylamino)-2-pyrimidinone 5'-Di- and Triphosphates (IIf, h). A total of 1.5 ml of 1 M IV with pH 5 was added to 0.2 ml of 1 M cytidine di- or triphosphate, and the mixture was incubated at 54° for 7-8 h and worked up as described above to give 20-25% (based on the starting cytosine derivative of pure IIf and IIh).

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