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Corresponding Member of the USSR Academy of Sciences N. K. Kochetkov, E. I. Budovskii,

N. A. Simukova

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Abstract

Full Text

CHEMISTRY

Corresponding Member of the USSR Academy of Sciences N. K. Kochetkov, E. I. Budovskii,
N. A. Simukova

PRIMARY STRUCTURE OF RNA

INTERACTION OF RNA WITH O-METHYLHYDROXYLAMINE

It was shown earlier in our laboratory that O-methylhydroxylamine (OMHA) reacts selectively with cytidine (optimum pH 6.0–7.0), without affecting the other nucleosides that are constituents of RNA. The reaction proceeds according to the scheme:

$$\text{cytidine derivative} + CH_3ONH_2 \longrightarrow (I) + (II)$$

I readily passes into II in acidic and alkaline media ([1]). In order to use this reaction for selective cleavage of RNA into blocks for the purpose of further establishing its primary structure (cf. ([2])), it was necessary: 1) to select conditions for selective and sufficiently complete modification of cytosine nuclei in the polymer chain of RNA, and 2) to determine whether, by means of this reaction, it is possible to increase the specificity of pancreatic ribonuclease (RNase) so that in RNA treated with OMHA the enzyme would cleave phosphodiester bonds only at uridine residues, without affecting the modified cytidine residues.

In Zillig' s opinion ([3]), the reaction of RNA with hydroxylamine should be hindered because of the secondary structure. According to the mechanism proposed by us ([1]), the presence of internucleotide hydrogen bonds should not affect the reaction of RNA with hydroxylamine and OMHA. The experimental data confirm this supposition. It was shown earlier that the reaction of cytidine with OMHA is practically complete after 72 hours ([1]). After incubation of RNA with 2 M OMHA, after only 24 hours (37°, pH 6.0), only insignificant amounts of unchanged cytidylic acid were found in the alkaline hydrolysate (Fig. 1).

These data show that practically all cytosine nuclei in RNA are modified, and the reaction previously studied for the nucleoside ([1]) is also applicable to the polymer. This conclusion is definitively confirmed by the result obtained in the interaction of 2 M OMHA with polycytidylic acid, which under analogous conditions (pH 6, 37°) is completely changed after 72 hours. The absence in it of unchanged cytosine nuclei is confirmed by the results of hydrolysis with pancreatic RNase (see below).

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It should be noted that, under the reaction conditions, in the case of polycytidylic acid no formation of low-molecular-weight substances was observed. For RNA, their amount after the reaction does not exceed 3–4% (gel filtration on Sephadex G-25 by optical density at 260 mµ).

Thus, the reaction with OMHA is the first reaction that makes it possible to modify the cytosine nucleus in nucleic acids strictly selectively. Owing to the mildness of the reaction conditions, it is evidently suitable for modi-

fication of biologically active polynucleotides (t-RNA, DNA, etc.), which is of undoubted interest for genetic and biochemical studies.

For the application of this reaction for the purpose of selective cleavage of the RNA chain, we proposed to use pancreatic RNase, which, as is known, cleaves phosphodiester bonds between a pyrimidine nucleoside 3′-phosphate and the neighboring nucleotide in two stages: 1) cleavage of the 3′,5′-phosphodiester bond with formation of a 2′,3′-cyclophosphate, 2) cleavage of the cyclophosphate bond with formation of a 3′-phosphate.

In a previous work [4] we successfully used a method for increasing the specificity of RNase by chemical modification of RNA. It was necessary to verify whether the changes caused by OMHA in the cytosine nucleus are sufficient to block the substrate specificity of the enzyme.

Despite the considerable attention currently being paid to the question of the mechanism of action and specificity of RNase, it still remains unclear what changes in the pyrimidine base are necessary and sufficient for blocking the substrate specificity of RNase [5]. There are data indicating blocking of the action of RNase upon substitution at N_3 in the pyrimidine nucleus [6,7].

Fig. 1. Chromatography of an alkaline hydrolysate of RNA (a) and of RNA treated with O-methylhydroxylamine (b). Anion exchanger Dowex 1 × 4, 200–400 mesh, Cl^- form, 0.5 × 3 cm, elution as shown in the figure. a –10 mg of RNA are dissolved in 2 ml of 1 *N* KOH, incubated for 24 hours at 37°, diluted to 20 ml, treated with cation exchanger IRC 50 in the H^+ form to pH 8, and applied to the column. b –13 mg of RNA are dissolved in 2.3 ml of 2 *N* O-methylhydroxylamine, pH 6.0, incubated for 24 hours at 37°, applied to a Sephadex G-25 column (1.5 × 50 cm). They are eluted with water. Fractions containing the polymer are lyophilized, hydrolyzed with alkali as indicated in (a), and applied to the column.

Witzel proposes that, in order to preserve substrate specificity, the presence of an intact grouping [8] is necessary

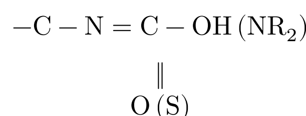

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Fig. 2. Chromatography of RNase hydrolysates of RNA (a) and polycytidylic acid (b), treated with O-methylhydroxylamine.

Figure 1: Fig. 2. Chromatography of RNase hydrolysates of RNA (a) and polycytidylic acid (b), treated with O-methylhydroxylamine.

It has been shown that hydrogenation of pyrimidine does not affect the action of RNase [(9)].

In light of these data, the problem we posed was also of interest from the standpoint of studying the mechanism of action and specificity of pancreatic ribonuclease.

The usual model for studying substrate properties with respect to RNase is nucleoside 2′,3′-cyclophosphates.

We have shown that treatment of cytidine 2′,3′-cyclophosphate with 2 M OMHA (37°, pH 6.0, 48 h) leads to the formation of two cyclophosphates (III and IV), corresponding to I and II.

The structure of III and IV is confirmed by spectral data and by electrophoretic mobility at pH 7.5, indicating preservation of the cyclophosphate grouping. Upon incubation of III and IV with RNase, the cyclophosphate grouping is opened with formation of the corresponding 3′-phosphates.

Thus, at least for blocking the second stage of RNase action, the indicated changes in the cytosine nucleus are insufficient. Taking into account data on the possibility that the two stages of hydrolysis of the phosphodiester bond are carried out by different active centers of RNase (^10), it could be supposed that the first stage would prove more sensitive to modification of the pyrimidine nucleus.

To test this assumption, cleavage by pancreatic RNase of high-polymeric RNA with modified cytosine nuclei was studied. Ion-exchange chromatography of ribonuclease hydrolysates of RNA before and after treatment with OMHA according to Bell, Tomlinson, and Tener (^11) showed that the amounts of mono-, di-, tri-, etc. oligonucleotides in both cases are practically identical. During chromatography on Dowex-1 of the mononucleotide fractions from a ribonuclease hydrolysate of RNA treated with OMHA, uridylic acid and O-methoxyuridylic acid were detected. This shows that RNase cleaves the polynucleotide chain both at uridine and at modified cytidine residues, i.e., in this case it does not display a narrower specificity.

Fig. 2. Chromatography of RNase hydrolysates of RNA (a) and polycytidylic acid (b), treated with O-methylhydroxylamine. Anion-exchanger DEAE-Sephadex A-25, medium, Cl^{-} form, 0.7 × 12 cm. Chromatography is carried out in 7 M urea solution containing 0.005 M Tris (pH 7.8). Elution: linear gradient of sodium chloride. a –10 mg of RNA is treated with O-methylhydroxylamine, as indicated in Fig. 16. The dry substance, obtained

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after lyophilization, is dissolved in 1.2 ml of 0.02 M phosphate buffer (pH 7.6), incubated with 1 mg of RNase for 24 h at 37°. The hydrolysate is diluted to 40 ml with 7 M urea solution containing 0.005 M Tris (pH 7.8), and applied to the column. b −1 mg of polycytidylic acid is dissolved in 0.2 ml of 2 M O-methylhydroxylamine, pH 6.0, incubated for 72 h at 37°, applied to a Sephadex G-25 column, 78 × 1.4 cm, equilibrated with 0.01 M Tris-HCl (pH 6.0). Elution is carried out with the same buffer. Fractions containing polymer are lyophilized, incubated with RNase, as indicated in (a), and applied to the column.

These data were definitively confirmed in the study of ribonuclease hydrolysis of polycytidylic acid subjected to preliminary treatment with OMHA (see above). Upon incubation of such polycytidylic acid with ribonuclease, complete cleavage to monomers occurs.

In ion-exchange chromatography according to Bell, Tomlinson, and Tener [(11)], only the mononucleotide fraction was isolated (Fig. 2), identified by its UV spectrum and by paper chromatography (cf. [(1)]) as *O*-methoxyurididylic acid. The latter, incidentally, proves that when polycytidylic acid reacts with OMHA, all cytosine nuclei are modified. Thus, modification of the cytosine nucleus in RNA by the action of OMHA is insufficient to make the action of pancreatic ribonuclease more specific. Evidently, such a serious change in the pyrimidine nucleus as the conversion of an amino(imino) group into an *O*-methyloxime group does not affect the specificity of the enzyme. It would be interesting to test, for the same purposes, other substituted hydroxylamines with bulkier substituents. These possibilities are now being investigated by us.

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Laboratory of Carbohydrates and Nucleotides
Institute of Chemistry of Natural Compounds
Academy of Sciences of the USSR

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SovietRxiv

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