DETERMINATION OF THE NUMBER OF CYTIDINE RESIDUES IN OLIGONUCLEOTIDES

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1. Introduction

The present communication describes a principle of determination of the number of given type nucleoside residues in oligonucleotides. The principle is based upon nucleoside-specific chemical modification resulting in a change of the charge of the nucleoside residue. Using the methods based on this principle, it is enough to find the number of oligonucleotides of different extents of modification present in the reaction mixture instead of making accurate quantitative analyses to find the length of the oligonucleotide and its nucleoside composition.

The principle is exemplified by a procedure for the determination of the number of cytidine residues in oligonucleotides involving modification of cytidine residues with a mixture of *O*-methylhydroxylamine and bisulfite. The modification results in negatively charged N^4-methoxy-5,6-dihydro-6-sulfonate residues [1].

2. Materials and methods

Preparation of the modifying reagent, *O*-methylhydroxylamine-bisulfite mixture, has been described earlier [2]. Pentanucleotides of the general formula pPypPypPypPypPyp were obtained by hydrolysis of T7 phage DNA according to Burton and Peterson [3] followed by fractionation on DEAE-cellulose in 7 M urea at pH 5.5 [4]. The pentanucleotides were subsequently rechromatographed on DEAE-cellulose in 7 M urea at pH 3.7 [5] to obtain mixtures of isomers of different C content. The mixtures designated as T_4C, T_3C_2, T_2C_3 were used for the following experiments.

The optical densities of effluents were recorded by a microspectrophotometer designed at the Institute of Organic Chemistry (Novosibirsk). The volume of the flow-cell of this instrument is 2 μl [6].

In a typical modification experiment, 10 μl (about 0.3 A_{260} units) of the pentanucleotides, 8 μl of 5 M *O*-methylhydroxylamine, pH 6.0 and 20 μl of 2 M sodium metabisulfite, pH 6 were thoroughly mixed and kept at 20°. 10 μl aliquots were withdrawn in 0.5, 1, 1.5 and 2 hr and mixed together in a teflon cup stored at −5°. After addition of the last aliquot, the mixture was desalted on a 600 μl, 20 cm long Sephadex G-10 column in bidistilled water. The following fractionation of modified oligonucleotides was performed by means of micro-column ion-exchange chromatography techniques developed in the Novosibirsk Institute of Organic Chemistry [4, 5]. The desalted mixture was applied to a 50 μl, 10 cm long DEAE cellulose (for TLC, Serva, BRD) column. Elution was performed by 600 μl linear gradient of sodium chloride (0 to 0.3 M) in 0.01 N NaOAc, 7 M urea, pH 5.5 at flow rate 150 μl/hr (see fig. 1).

3. Results and discussion

One could argue that to determine the number of cytidine residues in an oligonucleotide it would be enough to modify its C residues completely with *O*-methylhydroxylamine-bisulfite and to determine the change of the net charge caused by the modifica-

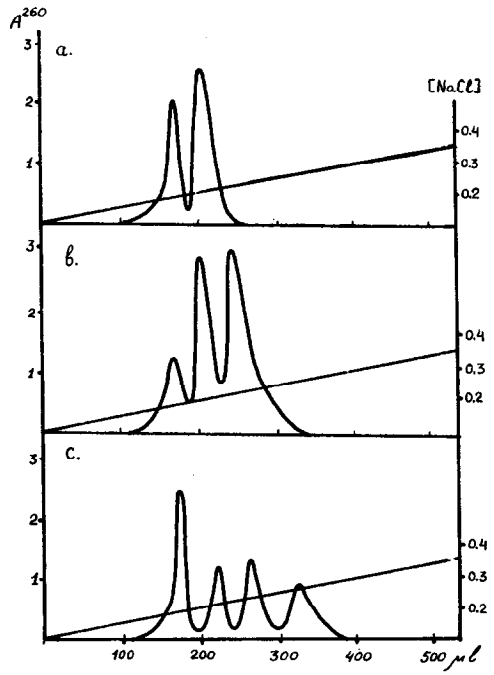

Fig. 1. Chromatographic separation of the combined reaction mixture aliquots after partial modification of cytidine residues in pyrimidine pentanucleoside hexaphosphates with *O*-methylhydroxylamine-bisulfite. a) T_4C; b) T_3C_2; c) T_2C_3. The designations and the conditions are described under Materials and methods.

tion. However, it is not a simple task to determine exactly the net charge of such a completely modified oligonucleotide since fractionation methods like ion-exchange chromatography in 7 M urea at neutral pH or electrophoresis which could be used to this end are sensitive to other properties besides the net charge of the molecule. For this reason we preferred to determine the number of cytidine residues more directly, by complete resolution of the modified oligonucleotides present in the reaction mixture according to the extent of their modification. Obviously, from the starting oligonucleotide containing n cytidine residues, the reaction mixture after incomplete modification will contain n+1 products differing by the extent of modification, including the unmodified species. As expected, it appeared simple to resolve all these products according to their charge by chromatography at neutral pH in 7 M urea.

Evidently, the relative amounts of the products of different extent of modification change in the course of reaction. For this reason the concentrations of some of the species after a given time interval could appear below the detectable level because they had not yet accumulated or, vice versa, were transformed to more extensively modified products. To overcome this difficulty, we analyzed the mixture obtained by pooling together aliquots withdrawn at different times from the very beginning of the reaction till practically complete modification of all the cytidine residues.

The application of the method is illustrated by determination of the number of cytidine residues in pyrimidine pentanucleoside hexaphosphates obtained by treatment of DNA with diphenylamine-formic acid according to Burton and Petersen [3]. The aliquot mixture was prepared as described under Materials and methods and fractionated by micro-column ion-exchange chromatography [5, 6] on DEAE-cellulose in 7 M urea at pH 7.5. The fractionation patterns are shown in fig. 1. It is seen, that the T_4C isomer mixture (for designations see Materials and methods) gives two peaks which obviously correspond to the unmodified and to the mono-modified species. As expected, three peaks were found in the case of T_3C_2 (unmodified, mono-modified, bi-modified), and four peaks in the case of T_2C_3 (unmodified, mono-modified, bi-modified, tri-modified).

The maximum time of reaction used in the present studies was 2 hr. One must increase the time of modification with longer oligonucleotides, but 3 hr will be enough to achieve practically complete modification of oligonucleotides containing up to 8 cytidine residues.

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