QUANTITATIVE DETERMINATION OF THE ACETYL GROUPS IN NUCLEOTIDE ACETATES

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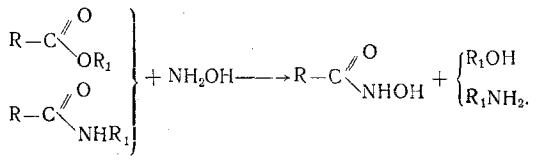
In the chemical synthesis of oligonucleotides, acetylation is widely used to protect the reactive groups. It is quite natural for the question to arise of the quantitative evaluation of the degree of substitution of the acetate obtained. It is particularly important to have available a method for determining acetyl groups in order to characterize the acetates of dinucleotide blocks in connection with their use as the initial components in the synthesis of higher oligonucleotides [1]. Micromethods of determining acetyl groups, based on the saponification of the acetates with alkali and the distillation of the acetic acid liberated, that are known in the literature are unsuitable for nucleotides and particularly to oligonucleotides, if only because the amount of substances taken for one sample is 10 mg. Synthetic work with oligonucleotides is frequently carried out at this quantitative level. Furthermore, these methods are insufficiently universal [2].

The present paper describes a spectrophotometric method for determining acetyl groups which is based on the reaction of derivatives of organic acids with hydroxylamine in the presence of alkali, which takes place in the following way:



The hydroxamic acid produced in the reaction forms a colored complex with salts of tervalent iron. The intensity of the coloration of a solution of the complex at 535 nm is directly proportional to its concentration [3]. This reaction has been used previously for the analysis of acetyl groups in RNA and polynucleotides acetylated at the 2'-hydroxyl [4]. We are the first to have applied this method to the characterization of dinucleotides acetylated at all the OH and NH_2 groups. For this purpose, the molar concentrations of O–Ac and N–Ac groups in a solution of the substance being analyzed were determined by the hydroxamate method. To calculate the degree of substitution of the acetate, the figure obtained must be referred to the molar concentration of nucleotide determined by the spectrophotometric method after the saponification of the acetyl groups in a sample of the solution under analysis. The alkaline hydrolysis of O–Ac groups by aqueous ammonia takes 10-20 min. The N–Ac groups hydrolyze more slowly (5-10 h). The group most difficult to hydrolyze is the N–Ac group of the guanine nucleus, the saponification of which requires ~20 h.

We have developed a more rapid and convenient method for determining the concentration of the nucleotide in the initial solution using the conditions of the first stage of the hydroxamate method of analysis (before the formation of the Fe^{+++} complex). The hydroxamic acid present in the acid solution does not affect the spectrum of the nucleotide [5].

Under suitable conditions, hydroxylamine reacts with the heterocyclic bases uridine and cytidine; the purine bases remain unaffected [6, 7]. The reaction conditions mentioned are fairly severe: elevated temperature, high concentration of hydroxylamine, long reaction time. The spectra of uridine and cytidine 2'(3')-phosphates undergo no change under the conditions of their hydroxamate analysis, and the spectra of the products of the reaction of hydroxylamine with an acetate differ from the spectrum of the hydrolyzed acetate (Fig. 1a, b) at 260 nm, i.e., in the region in which it is most convenient to measure the concentration

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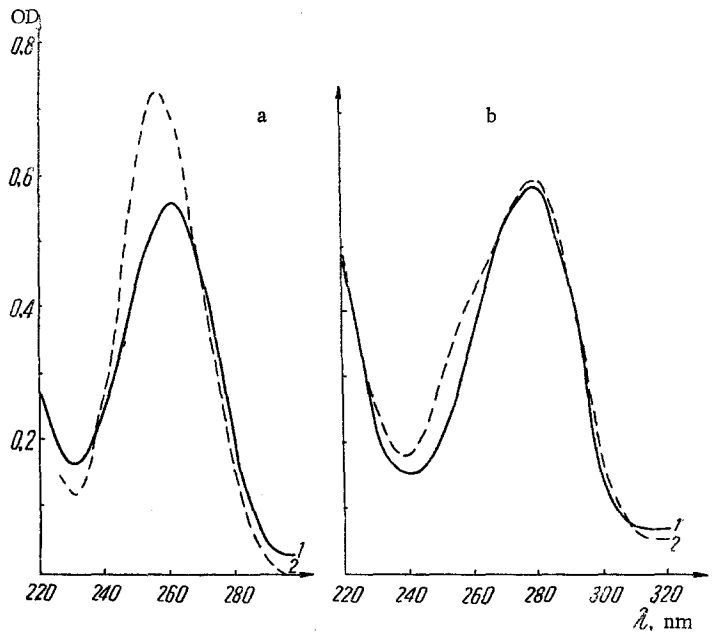

Fig. 1. UV spectra of uridine 2'(3')-phosphate obtained after the alkaline hydrolysis of the Ac groups (1) and the product of the reaction of the diacetate with NH_2OH (2) (a); cytidine 2'(3')-phosphate, liberated by the alkaline hydrolysis of the Ac groups (1) and the product of the reaction of the triacetate with NH_2OH (2) (b).

TABLE 1. Determination of the Content of Acetyl Groups in the Acetates of Uridine 2'(3')-Phosphate and Cytidine 2'(3')-Phosphate

Substance	OD at 535 nm	Amt* of Ac groups, μmole/ml	Results of alkaline hydrolysis of the Ac groups				Results of reaction with hydroxylamine				
			B, ml	OD_{260} nm	c, μmole per ml	Degree of substitut.	B, ml	OD_{270} nm (calc.)	OD_{260} nm (calc.)	c, μmole / ml	Degree of substitut.
Diacetate of uridine 2'(3')-phosphate $\varepsilon_{260\,nm}\cdot10^{-3}=10{,}0$ $\frac{OD_{270\,nm}}{OD_{260\,nm}}=0{,}82$ [8]	0,465	1,86	0,2	0,640	0,961	1,94	0,2	0,518	0,631	0,946	1,97
	0,420	1,68	0,2	0,562	0,844	1,99	0,2	0,425	0,518	0,775	2,17
Triacetate of cytidine 2'(3')-phosphate $\varepsilon_{260\,nm}\cdot10^{-3}=6{,}8$ $\frac{OD_{270\,nm}}{OD_{260\,nm}}=1{,}51$ [8]	0,555	2,25	0,2	0,345	0,760	2,96	0,2	0,530	0,350	0,770	2,93
	0,760	3,07	0,2	0,490	1,08	2,84	0,2	0,745	0,493	1,09	2,82

*Results taken from the calibration curve (see Experimental).

of the nucleotide. In a detailed examination of the spectra, it was found that the optical densities of the saponified nucleotides and the products obtained after the reaction of hydroxylamine are the same at 270 nm. Consequently, the optical density of the solution of the initial nucleotide at 260 nm can be calculated by using the ratio of the optical densities $OD_{270\,nm}/OD_{260\,nm}$ known from the literature [8]. Then the concentration of nucleotide (c, μmole/ml) can be calculated from the formula

$$c=\frac{OD_{270\,nm}A}{\frac{OD_{270\,nm}}{OD_{260\,nm}}\cdot B\cdot\varepsilon_{260\,nm}10^{-3}},$$

where A is the volume of the solution measured (hence A = 3 ml); B is the volume of the sample of the initial solution taken for analysis, ml; and $\varepsilon_{260\,nm}$ is the molar extinction of the initial nucleotide at 260 nm.

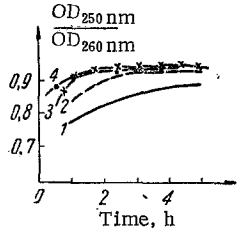

Fig. 2

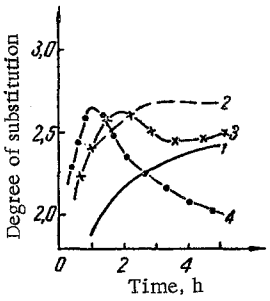

Fig. 3

Fig. 2. Dependence of $OD_{250\,nm}/OD_{260\,nm}$ on the time of the reaction of the triacetate of guanosine 2'(3')-phosphate with NH_2OH: 1) at 20°C; 2) at 40°C; 3) at 50°C; 4) at 60°C.

Fig. 3. Dependence of the degree of substitution found for the acetate of guanosine 2'(3')-phosphate on the time of the reaction with NH_2OH: 1) at 20°C; 2) at 40°C; 3) at 50°C; 4) at 60°C.

TABLE 2. Determination of the Content of Acetyl Groups in the Triacetate of Adenosine 2'(3')-Phosphate, $\varepsilon_{260\,nm} \cdot 10^{-3} = 14.2$ [8]

Method	$OD_{535\,nm}$	Amt. of Ac groups, μmole/ml	B, ml	$OD_{260\,nm}$	c, μmole/ml	Degree of substitution
Reaction with NH_2OH	0,460	1,84	0,1	0,310	0,655	2,81
Alkaline hydrolysis of the Ac groups	—	—	0,18	0,530	0,622	2,96
Reaction with NH_2OH	0,715	2,88	0,1	0,467	0,988	2,92

TABLE 3. Determination of the Content of Acetyl Groups in the Triacetate of Guanosine 2'(3')-Phosphate, $\varepsilon_{260\,nm} = 11.8$ [8]

Time, min	$OD_{535\,nm}$	Amt. of Ac groups, μmole/ml	Results of alkaline hydrolysis of Ac groups			
			B, ml	$OD_{260\,nm}$	c, μmole/ml	Degree of substitution
10	0,440	1,76	0,14	0,497	0,902	1,95
30	0,475	1,91	0,14	0,497	0,902	2,12

This method of calculation has given completely satisfactory results (Table 1). The deviations in the calculated degrees of substitution from the theoretical values do not exceed 8.5%. Thus, in the case of an individual acetate it is possible to judge its structure with a fair degree of certainty.

The $OD_{260\,nm}$ value of the triacetate of adenosine 2'(3')-phosphate was measured immediately after the reaction with NH_2OH (Table 2).

It can be seen from Table 3 that under the usual conditions of analysis, only two acetyl groups are determined in the acetate of guanosine 2'(3')-phosphate. In determining the concentration of nucleotide by means of the reaction of hydroxylamine, a still lower degree of substitution was obtained, which showed the unsuitability of the spectra of guanosine 2'(3')-phosphate and of the reaction product. The completeness of the elimination of the acetyl groups from the nucleotide can be judged from the dependence of the ratio of the optical densities at 250 and 260 nm on the time of reaction of the acetate with hydroxylamine at various temperatures (Fig. 2). For guanosine 2'(3')-phosphate, $OD_{250\,nm}/OD_{260\,nm} = 0.94$ [8]. At 20°C, the curve does not reach the theoretical value in 5 h, and at elevated temperatures the curves reach this value in different times. A different pattern is observed if we consider the dependence of the degrees of substitution determined on the time of the reaction at various temperatures (Fig. 3). In no case did the degree of substitution reach the limiting value, and at 50 and 60°C at a definite stage of the reaction it fell sharply. This

TABLE 4. Characteristics of the Triacetate of Adenosine 2'(3')-Phosphate

Substance	R_f	R_m	λ_{max} nm	Notes
Adenosine 2'(3')-phosphate	0,16	1	258	Found
Triacetate of adenosine 2'(3')-phosphate	0,56	0,73	272	
Adenosine 3'-phosphate	0,21	1	258	Literature data [9, 10]
N,2',5'-O Triacetyl adenosine 3'-phosphate	0,59	0,68	272	

is probably due to the fact that the acethydroxamic formed in the first stages of the reaction decomposes with time the more rapidly the higher the temperature [5]. In experiments at temperatures above 60°C the complete liberation of the guanosine 2'(3')-phosphate took place in 10-20 min and the degree of substitution did not exceed 2.3. Summarizing, it may be concluded that the optimum conditions for determining the acetyl groups in the triacetate of guanosine 2'(3')-phosphate are a temperature of 40°C and a time of 3 h, and the deviation from the theoretical value of the degree of substitution amounts to 10%.

EXPERIMENTAL

Materials and Methods. The acetates of the mononucleotides were obtained by acetylating the pyridinium salts of mixtures of isomers of uridylic, cytidylic, adenylic, and guanylic acids by Khorana's method [9, 10]. The pyridinium salts of the compounds mentioned, in their turn, were prepared from the corresponding reagents of the firm of "Reanal" (Hungary) by passage through Dowex-50 resin in the pyridinium form. Reagents of "pure for analysis" grade, recrystallized from water, were used to prepare the solutions of hydroxylamine hydrochloride and iron sulfate.

The optical density at 535 nm was measured in an SFD-2 spectrophotometer, and the UV spectra on an SF-4 spectrophotometer.

Determination of Acetyl Groups. To 1 ml of a solution of a nucleotide acetate containing 2-3 μmole of acetyl groups in 50% aqueous ethanol was added 1 ml of 0.4 M hydroxylamine hydrochloride and 0.5 ml of 1 M caustic soda solution. The reaction mixture was kept for 10 min and then 0.5 ml of 0.1 M solution of $Fe_2(SO_4)_3$ in 2 N H_2SO_4 was added, and the optical density of the solution of the hydroxamate complex was measured at 535 mμ.

Determination of the Concentration of Nucleotide. To analyze the concentration of nucleotide, samples were taken which contained about 1.5 optical units of material, and their volume was brought up to 1 ml with 50% aqueous ethanol, except that, in place of the solution of iron sulfate, 2 N H_2SO_4 in the same volume was added.

Hydrolysis of the Acetyl Groups. A sample of the nucleotide material with an absorption at 260 nm of about 1.5 was placed in a round-bottomed flask with a volume of 5-10 ml. The solvent was evaporated in vacuum without heating, and the residue was hydrolyzed with conc. ammonia (0.2-0.5 ml) overnight. Then the ammonia was driven off in vacuum, and the dry residue was dissolved in 0.3 ml of 0.1 N HCL, the optical density of the solution at 260 nm was measured, and the molar concentration of nucleotide was calculated.

Construction of the Calibration Curve. The triacetate of adenylic acid, identified by paper chromatography in the ethanol–1 M NH_4OCOCH_3 system, pH 7.5 (7 : 3), by paper electrophoresis in 0.03 M phosphate buffer (pH 7.1), and by UV spectroscopy (Table 4) was used as the standard.

The content of acetyl groups was calculated from the amount of adenylic acid in solutions of the triacetate in 50% ethanol after alkaline saponification of the acetate on the assumption that there are 3 moles of acetyl groups per mole of nucleotide.

CONCLUSIONS

1. A spectrophotometric method for the quantitative determination of the acetyl groups in acetates of mononucleotides by means of the acid hydroxamic complex with $Fe_2(SO_4)_3$ has been developed. It has been shown that the determination of the concentration of nucleotide material in the solution being analyzed can be carried out directly in the reaction mixture in the first stage of the reaction of the acetate with hydroxylamine (before the formation of the Fe^{+++} complex).

2. In the case of uridine, cytidine, and adenosine derivatives, the reaction with hydroxylamine takes place quantitatively in 10 min at 20°C, while for the determination of the acetyl groups in the triacetate of guanosine 2'(3')-phosphate the reaction was carried out at 40°C for 3 h.

LITERATURE CITED

1. N. S. Tikhomirova-Sidorova, G. E. Ustyuzhanin, A. P. Kavunenko, E. M. Kogan, and E. A. Pyaivinen, "The synthesis, structure, and properties of polymers," Transactions of the XV Scientific Conference of the Institute of High-Molecular Weight Compounds, AS USSR [in Russian] (1970), p. 201.
2. S. N. Tabib, S. Y. Kulkarni, and V. S. Pansare, Microchem. J., 13, 986 (1968).
3. Biochem. Prep., 8, 14.
4. D. G. Knorre, N. M. Pustoshilova, N. M. Teplova, and G. G. Shamovskii, Biokhim., 30, 1218 (1965).
5. N. K. Kochetkov, É. I. Budovskii, and V. D. Domkin, Mol. biol., 1, 558 (1967).
6. H. Schuster, Mol. biol., 3, 447 (1961).
7. N. K. Kochetkov, É. I. Budovskii, and N. A. Simukova, Biokhim., 27, 519 (1962).
8. T. V. Venkstern and A. A. Baev, Absorption Spectra of the Minor Components and Some Oligonucleotides of Ribonucleic Acids [in Russian], Moscow (1967).
9.*
10. Y. Lapidot and H. G. Khorana, J. Am. Chem. Soc., 85, 3852 (1963).
11. R. Lhormann and H. G. Khorana, J. Am. Chem. Soc., 86, 4188 (1964).

* Missing in Russian original – Publisher.