ALKYLATION OF NUCLEIC ACIDS AND THEIR COMPONENTS

V.* REACTION OF N-β-CHLOROETHYL-N-METHYL-1,3-PROPYLENEDIAMINE WITH GUANOSINE AND TRANSFER RNA

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N-β-Chloroethyl-N-methyl-1,3-propylenediamine alkylates guanosine and the guanine in transfer RNA to give 7-[β-(N-γ-aminopropyl-N-methylamino)ethyl]guanosine and the similarly alkylated transfer RNA. The alkylation of guanosine with excess reagent is accompanied by quaternization of the tertiary amino group of the substituent entering into the guanosine with a subsequent molecule of reagent. In acid, 7-[β-(N-γ-aminopropyl-N-methylamino)ethyl]-guanosine is converted to 7-[β-(N-γ-aminopropyl-N-methylamino)ethyl]guanine, while in alkali it is converted to 2-amino-5-[β-(N-γ-aminopropyl-N-methylamino)ethylformamido]-6-ribofuranosylamino-4-pyrimidinone. Hydrolysis of the latter in acid gave 5-[β-(N-γ-aminopropyl-N-methylamino)ethylformamido]-2,6-diamino-4-pyrimidinone. 7-[β-(N-γ-Aminopropyl-N-methylamino)ethyl]guanine was isolated from the acid hydrolyzate of the alkylated transfer RNA.

The carcinogenic and mutagenic properties of nitrogen mustards and 2-chloroethylamines have stimulated the investigation of the chemistry of the alkylation of nucleic acids by these reagents [2]. Nitrogen mustards are also of interest as bifunctional reagents for the study of the conformations of nucleic acids [2, 3]. In addition, alkylation with 2-chloroethylamines is a method for the introduction of basic substituents into nucleic acids; this can be used for the study of the structures of nucleic acids by means of electron microscopy [4].

With the aim of developing methods for the specific modification of nucleic acids [5], we studied the alkylation of guanosine (I) and transfer RNA with N-β-chloroethyl-N-methyl-1,3-propylenediamine (II). We determined the pK_a of II in water at 25°C; it is 8.8 for the γ-amino group and 6.6 for the trialkylamino group. As an aliphatic 2-chloroethylamine [6], II in aqueous media is converted to N-(γ-aminopropyl)-N-methylethyleneimmonium chloride (III). Judging from the iodimetric titration of excess thiosulfate in samples of solutions of II with pH ≥ 6, ~40% of III accumulates during the complete ionization of the chlorine, probably because of consumption of a portion of III in the reaction with II [7]. At pH 5.0, ionization of the chlorine in II proceeds at a constant rate $(k_{obs})^{\dagger}$ of $1.07 \cdot 10^{-4}$ sec^{-1} (25°); hence, $k_{true}^{\dagger}$ is $4.4 \cdot 10^{-3}$ sec^{-1} (for pK_a 6.6). Up to 100% of the III accumulates at pH 5.0 during complete ionization of the chlorine. Compound III reacts slowly with water ($k_{H_2O}^{\dagger} = 2.5 \cdot 10^{-6}$ liter/mole · sec at 40° and pH 5.0).

At pH values above 6.5, II is rapidly converted to III, but it does not react with I under these conditions and is consumed primarily in the alkylation of II (see [7]). Alkylation of I is observed at pH 5.0-6.0 to give the unstable 7-[β-(N-γ-aminopropyl-N-methylamino)ethyl]guanosine (IV). Unambiguous evidence for the structure of IV is its conversion to 7-[β-(N-γ-aminopropyl-N-methylamino)ethyl]guanine (V) and ribose on reaction with acid and to 2-amino-5-[β-(N-γ-aminopropyl-N-methylamino)ethylformamido]-6-ribo-

* See [1] for communication IV.

† These values were determined by N. D. Kobets.

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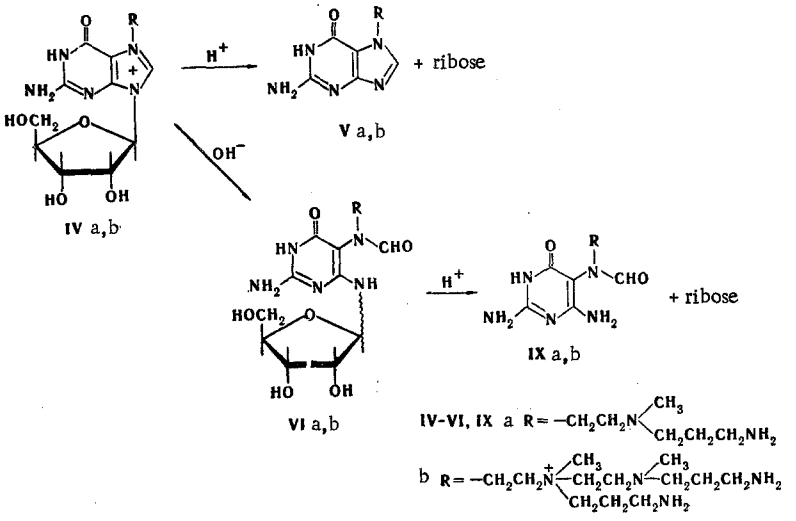
TABLE 1. UV Spectra of Products of the Alkylation of Guanosine

Compound	pH	λ_{max} ($\lambda_{shoulder}$), nm	$\varepsilon_{max} \cdot 10^{-3}$	λ_{min}, nm	$\varepsilon_{260} \cdot 10^{-3}$
Va	1	249(270)	10,65	230	8,5
	7	248; 283	9,0; 9,12	260	—
	13	280	9,5	262	—
Va from transfer RNA	1	250(270)	—	230	—
	7	248; 285	—	260	—
Vb	13	280	—	262	—
7-Methylguanine [9]	1	250(270)	10,96	228	8,2
	6	248; 283	—; 7,41	260	—
	13	280	7,76	257	—
IXa, IXb	1	263	18,0	240	17,3
	7	262	10,7	240	—
	13	260	8,2	235	—
VI	1	270	19,8	245	13,0
	6	272	18,3	247	—
	13	265	13,2	243	—
VIII [13]	1	269	18,1	240	—
	12	265	11,8	242	—
VII [9]	1	258(277)	—	229	—
	7	258; 281	—	271; 238	—
	9,2	283	—	242	—

furanosylamino-4-pyrimidinone (VI) in alkaline media. This is analogous to the conversions of 7-methylguanosine (VII) [8]. The UV spectrum of IV is identical to the UV spectrum of VII (Table 1). It should be noted that opening of the ring in IV proceeds more readily than in VII [2, 8]. Compound IV is converted to VI at pH 6 (40°) at an appreciable rate. If alkylation of I is carried out at pH 5-6, the resulting IV has time after 4 h of reaction to be converted (15%) to VI. Compound IV cannot be isolated in pure form; after separation on DEAE-Sephadex in ammonium acetate at pH 3.7, impurity VI is detected in the IV fraction; primarily VI is obtained during paper chromatography in isopropyl alcohol–0.01 M ammonium acetate (6:4) at pH 3.7 or on DEAE-Sephadex in water.



Like the corresponding methyl analog (VIII) [8], VI has the same UV spectrum at pH 2-13; on reaction with acids, it readily loses ribose and gives 5-[β-(N-γ-aminopropyl-N-methylamino)ethylformamido]-2,6-diamino-4-pyrimidinone (IX). However, the formyl group in IX is hydrolyzed under more severe conditions than that in 5-N-methylformamido-2,6-diamino-4-pyrimidinone (X) [10]. It is retained in IX after refluxing in 1 N HCl and on standing in 6 N HCl.

In order to judge the alkylation of I without complications associated with the instability of IV, the alkylation products were separated after conversion to IV, V, or IX. For this, the reaction mixture was hydrolyzed by refluxing in xylene immediately after alkylation (1) or after alkaline treatment (2) and chromatographed on Dowex-50 in a linear gradient of hydrochloric acid. The chromatograms of the separation of the reaction mixtures are presented in Figs. 1 and 2 for cases (1) and (2). Two or more fractions of alkylated substances are formed in the alkylation with excess II. The fractions obtained were chromatographed repeatedly on paper and it was observed that each fraction of the alkylated derivatives contains a mixture of V and IX. Fraction 2 (Figs. 1 and 2) yielded Va and IXa, each of which contain one primary amino group

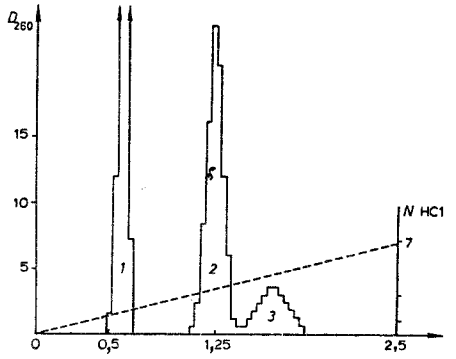


Fig. 1

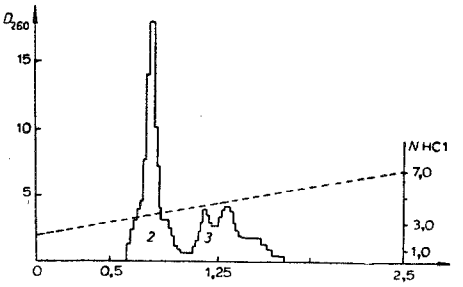


Fig. 2

Fig. 1. Chromatogram of the separation of the products of alkylation of guanosine II after acid hydrolysis via method (1) on Dowex-50 (H^+): 1) guanine; 2) mixture of Va and IXa; 3) mixture of Vb and IXb.

Fig. 2. Chromatogram of the separation on Dowex-50 of the products of alkylation of guanosine II after treatment with alkali and acid via method (2) (reaction of 5 mmole of I and 50 mmole of II): 2) mixture of Va and IXa; 3) mixture of Vb and IXb.

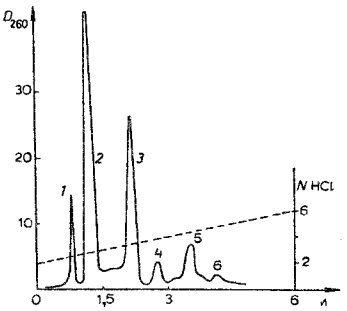


Fig. 3. Chromatogram of the separation on Dowex-50 (H^+) of a mixture of 5-N-alkylformamido-2,6-diaminopyrimidines (IX) obtained by hydrolysis of VI in acid [VI was obtained by alkylation of 5 mmole of I with 100 mmole of II for 24 h and was isolated by chromatography on DEAE-Sephadex A-25 (HCO_3^-)]: 2) IXa (R=1); 3) IXb (R=2).

(according to reaction with ninhydrin) and one R residue [from the ratio of the integral intensities of the protons in the PMR spectra, namely, the β-CH_2 and C_8—H protons for Va in $(CD_3)_2SO$ or the $>N$—CHO proton for IXa in D_2O, Table 2]. Fraction 3 yielded Vb and IXb, in each of which there were two $\overset{+}{N}H_3$ groups (Table 3). The UV spectra of Va and IXa are identical to the spectra of Vb and IXb, respectively. Only derivatives with the same number of R (or same number of charges) were detected in each fraction. From the overall number of fractions with identical UV spectra that were eluted with Dowex-50 with increasing hydrochloric acid concentration, it can be assumed that the number of substituents attached to guanosine may reach five in the presence of a 20-fold excess of II (Fig. 3, fractions 2-6).

The coincidence of the UV spectra of substances from different fractions and the addition to I of two residues with two free NH_2 groups attest to the fact that alkylation of I is accompanied by quaternization of the trialkylamino group of the entering substituent under the influence of a subsequent molecule of III. Alkylation of I by a dimerized molecule of II is unlikely, since the chloroethylamino group in β-chloroethyltrialkylammonium derivatives is of low reactivity, because it cannot react through the ethyleneimmonium cation.

The alkylation of I with a twofold excess of II for 4 h gives 5-10% Va. In the presence of a 10-fold excess of II, ~30% of the I is alkylated, 20% of which is derivatives with $R \geq 2$. In the presence of 20-fold II, ~50% of monoalkylated derivatives of the entire amount of V and IX is found.

The alkylation of transfer RNA was carried out in a 20 mM solution of II at pH 5 and 40°. After 1 h, we observed the precipitation of a portion of the transfer RNA from solution, probably because of the formation of the polyalkylammonium salt of transfer RNA. The precipitation of nucleic acids of polyamines was also observed in [11]. Compound Va was detected along with adenine and uridylic and cytidylic acids in the acid hydrolyzate of the alkylated transfer RNA. The degree of modification of the guanine in the transfer RNA was 47%.

The alkylation of guanosine and nucleic acids with aliphatic β-chloroethylamines and nitrogen mustards was previously [2] studied at pH 7-8 [12-14]. In [13], two substances with identical UV spectra and

TABLE 2. PMR Spectra of the Substances Obtained

Substance	Chemical shifts, δ, ppm (relative integral intensities)				
	solvent	H_8	H_{CHO}	H_β	$H_{N\text{-}CH_3}$
Va	D_2O	Rapidly exchanged [20]		2,35(2) Quintet	3,19(3) Singlet
Va	$(CD_3)_2SO$	9,35(1)		2,37(2)	
IXa	D_2O		8,22(1) Singlet	2,35(2) Quintet	3,14(3) Singlet
II	D_2O			2,0(2) Quintet	2,95(3) Singlet

TABLE 3. Characteristics of the Substances Obtained

Substance	Found per mole of heterocyclic base		Empirical formula	Ionic chlorine, %	
	ribose, mole	NH_2 groups, mole		found	calc.
Va*	—	1,04	$C_{11}H_{19}N_7O \cdot H_2O \cdot 3HCl$	27,6	27,1
Vb	—	2,25	$C_{17}H_{34}N_9O \cdot 5HCl$	34,3	31,5
VIa	0,96	—	$C_{16}H_{28}N_7O_6 \cdot 2HCl$		
IXa	—	0,93	$C_{11}H_{21}N_7O_2 \cdot 3HCl$	28,0	27,1
IXb	—	2,10	$C_{17}H_{36}N_9O_2 \cdot 5HCl$		

* After drying at 105° (1 mm) for 3 h, this compound had the following analytical values: Found: C 35.2; H 5.4; N 25.6%. $C_{11}H_{19}N_7O \cdot 3HCl$. Calculated: C 35.2; H 5.1; N 26.1%.

R_f values (elution with Dowex-50 in 4 N HCl) were obtained. Alkylation of DNA [3] gave, in addition to 7-monoalkylguanine, a derivative that was eluted in 6 N HCl, for which a structure of a 7-dialkyl derivative alkylated at the amino group of the substituent was proposed. On the basis of analogous data in [12], the products of the alkylation of thymine were identified as 3-dialkyl derivatives.

EXPERIMENTAL

Descending chromatography on Leningradskaya B and S papers was carried out with the following solvent systems: tert-butyl alcohol–methyl ethyl ketone–HCOOH–water (40:30:15:15) (1); isopropyl alcohol–concentrated HCl–water (170:41:39) (2); isopropyl alcohol–water (6:4), 20% ammonia in the gas phase (3); isopropyl alcohol–water (6:4) (4); tert-butyl alcohol–methyl ethyl ketone–concentrated ammonia–water (40:30:10:20) (5); ethanol–1 M ammonium acetate, pH 6.5 (5:2) (6); butyl alcohol–CH_3COOH–water (5:2:3) (7). Electrophoresis on paper was carried out in 0.04 M triethylammonium bicarbonate (TEAB) at pH 7.5-8.0 for 1 h at 40 V/cm. The solutions were concentrated at 35° (15 mm). The amounts of nucleosides and bases were determined from the absorption of their solutions at 260 nm and ε_{260} via the method in [8, 10, 15, 16]. The ribose content was determined by the orcinol method [17]. The aliphatic amines on the paper were developed by means of reaction with ninhydrin and in iodine vapors. The number of primary amino groups was determined from the reaction with ninhydrin via the method in [18], and the equivalent coefficient (ε_{500} $1.5 \cdot 10^3$ liters/mole · cm) was found from the reaction with γ-dimethylaminopropylamine. The PMR spectra of 10-15% solutions of the substances in $(CD_3)_2SO$, CD_3OD, and D_2O were recorded with a Varian A-56/60 spectrometer with tetramethylsilane as the external standard.

Transfer RNA from baker's yeast was obtained with an experimental device from the Novosibirsk Institute of Organic Chemistry, Siberian Branch, Academy of Sciences of the USSR. Compound I from the Reanal Company was used in the experiments. The dihydrochloride of II was obtained via the method in [5] from chromatographed γ-(N-β-hydroxyethyl-N-methylamino)propyl phthalimide [7].

The alkylation of a 5 mM solution of I was carried out at 40° and pH 5.0-6.0, which was maintained by the addition of 1 N KOH. The II concentration varied from 10 to 100 mM. After 4 h, the reaction mixture was worked up via method (1) or (2). Method (1): The mixture was acidified to pH 2 and evaporated to dryness, the residue was dissolved in 1 N HCl, and the solution was heated at 100° for 1 h; the solution was then diluted up to 0.1 N HCl and chromatographed on Dowex-50 (H^+). Method (2): the mixture was

TABLE 4. Properties of the Investigated Compounds

Compound	HCl concn. that elutes the substance from Dowex (H^+), N	E_f relative to pU*		R_f values in systems						
		pH 3,5	pH 7,5	1	2	3	4	5	6	7
Va	3,5	2,0	0,46	0,08	0,06	0,53	0,60	0,5	0,58	0,22
Vb	4,6	1,95	0,5	—	—	0,37	—	—	0,43	0,20
VI				0,06	—	0,46	0,63	0,4	0,55	—
IXa	3,5	1,95	0,54	0,12	0,17	0,5	0,55	—	0,40	0,20
IXb	4—5	—	—	—	—	—	—	—	0,31	0,20
Guanine	1,8	—	—	0,23	0,20	0,55	0,51	0,36	0,54	—

* E_f is the electrophoretic mobility.

made alkaline to pH 10, allowed to stand for 2 h, and evaporated; the residue was hydrolyzed in hydrochloric acid and chromatographed as in method (1).

7-[β-(N-γ-Aminopropyl-N-methylamino)ethyl]guanosine (IV). A reaction mixture from a 5 mM solution of I and a 10 mM solution of II, handled as indicated above, was acidified to pH 3.7 and chromatographed on DEAE-Sephadex A-25 in the CH_3COO^- form (35 by 1.6 cm); IV (10%) was eluted in the first fraction with 0.1 M CH_3COONa with pH 3.7. The properties are presented in Table 1. Compound VI was obtained after desalinization on Sephadex or chromatography with isopropyl alcohol—0.01 M CH_3COONH_4.

2-Amino-5-[β-(N-γ-aminopropyl-N-methylamino)ethylformamido]-6-ribofuranosylamino-4-pyrimidinone (VIa). A reaction mixture obtained as in the preceding experiment was passed through DEAE-Sephadex in the HCO_3^- form (60 by 3 cm) and washed out of the column with water. The first fraction was evaporated several times with aqueous triethylamine, dried thoroughly, and washed thoroughly with absolute ethanol and ether. The residue was dissolved in 25 ml of methanol, and an equivalent amount of HCl in methanol was added to it. The resulting oil was triturated with ethanol, and 190 mg of the solid (from 350 mg) was dissolved in 1 ml of methanol. The solution was mixed with 2 ml of dry silica gel, and the mixture was applied to a column packed with silica gel (12 by 1.6 cm) equilibrated with $CHCl_3$. The column was washed with 100 ml of $CHCl_3$—alcohol (1 : 1) and eluted with system 4. The first fraction was evaporated, and the residue was precipitated from methanol solution by the addition of ether. The precipitate was chromatographed on paper in system 3. A substance with R_f 0.46 was eluted with water and dried lyophilically. Compound VIa was reprecipitated from methanol into ether containing an equivalent amount of HCl; the yield was 36 mg.

7-[β-(N-γ-Aminopropyl-N-methylamino)ethyl]guanine (V). After alkylation of 0.6 mmole of I with 6 mmole of II, the mixture was worked up via method (1). The hydrolysis was carried out in 15 ml of 1 N HCl, and chromatography was performed on Dowex-50 (47 by 1.8 cm) in a linear gradient of HCl: the mixer was 1.25 liters of water, the reservoir was 1.25 liters of 7 N HCl, and the fraction volume was 10 ml (Fig. 1). Fractions 2 and 3 were evaporated with water several times, and the residue was chromatographed successively on paper in systems 7, 1, 6, and 3. The substances were eluted with water (the R_f values are given in Table 4), and 19% Va and 3.5% IXa were obtained from fraction 2, and 3.9% Vb and 1% IXb were obtained from fraction 3. The percentages of the substances in the fractions were determined from D_{260} and ε_{260} (Table 1). The degree of conversion of guanosine was 28%. For analysis, Va was additionally purified to remove salts on Dowex-50 (16 by 1.8 cm); the column was washed with 0.5 liter of 1 N HCl, and Va was then eluted with 4 N HCl. The fraction was evaporated to dryness, evaporated several times with water, and held over KOH at 1 mm. The precipitate was recrystallized from methanol—ether to give 40 mg of Va.

5-[β-(N-γ-Aminopropyl-N-methylamino)ethylformamido]-2,6-diamino-4-pyrimidinone (IX). The reaction mixture after alkylation of 0.6 mmole of I with 6 mmole of II was worked up via method (2). The chromatography was carried out on Dowex-50 as indicated above (Fig. 2). After separation on paper as indicated above for V, fraction 2 gave 14% IXa and 9% Va. Fractions 3 and 4 gave 7.5% IXb in a mixture with IX, where $R \geq 2$.

Compound IXa was obtained in the individual state after purification as indicated for Va. Compound IXa was also obtained by hydrolysis of 5 mg of VIa in 1 ml of a solution of hydrogen chloride in methanol at 4° for 16 h; the yield was quantitative.

The pK_a of II was determined potentiometrically by titration of a 0.01 M aqueous solution of the hydrochloride of II with 0.1 N NaOH at 25° in a stream of nitrogen. The titration time was 20 min. The graphically determined pK_a values of 6.6 and 8.8 are approximate because of the lability of II.

Alkylation of Transfer RNA. A total of 12 ml of a solution of transfer RNA containing 6.67 μmole of P per milliliter (a 2 mM solution of transfer RNA) was added to a solution of 0.8 mmole of II in 28 ml of water (a 20 mM solution of II), and the mixture was then held at 40° and pH 5.8-6.0 for 3 h and at pH ~ 5 for 24 h. The precipitate was separated, and the solution was hydrolyzed at 100° for 1 h in 1 N HCl and chromatographed on Dowex-50 as indicated above. Fractions containing uridylic and cytidylic acids, guanine, and adenine were obtained as in [19]. Compound Va was isolated from the fraction eluted in 4 N HCl. The percentages of guanine and Va found were 16% and 14%, respectively. The degree of alkylation of the guanine of transfer RNA was 47%. The acid hydrolyzate can also be separated on paper in system 2 in 60 h.

LITERATURE CITED

1. A. M. Belikova, N. I. Grineva, and G. G. Karpova, Khim. Geterotsikl. Soedin., 106 (1972).
2. P. D. Lawley, in: Progress in Nucleic Acid Research and Molecular Biology, Vol. 5, Academic Press, London (1966), p. 89.
3. J. Dockocil and Z. Sormowa, Coll. Czech. Chem. Commun., 30, 481 (1965).
4. B. P. Ulanov, A. M. Serebryanyi, and R. G. Kostyanovskii, Dokl. Akad. Nauk SSSR, 176, 474 (1967).
5. N. I. Grineva, T. S. Lomakina, and G. V. Shishkin, Zh. Obshch. Khim., 39, 668 (1969).
6. A. Ya. Berlin and M. Yaguzhinskii, Usp. Khim., 34, 1293 (1965).
7. N. I. Grineva and T. S. Lomakina, Khim. Geterotsikl. Soedin., 413 (1973).
8. P. D. Lawley and P. Brookes, Biochem. J., 89, 127 (1963).
9. J. A. Haines, C. B. Reese, and A. Todd, J. Chem. Soc., 1406 (1964).
10. S. Hendler, E. Furer, and P. R. Srinivasan, Biochemistry, 9, 4141 (1970).
11. J. P. S. Agrell and O. Heby, Exptl. Cell. Res., 50, 668 (1968).
12. C. C. Price, G. M. Gaucher, P. Koneru, R. Shibakawa, J. Sowa, and M. Yamaguchi, Biochim. Biophys. Acta, 166, 327 (1968).
13. P. Brookes and P. D. Lawley, J. Chem. Soc., 3923 (1961).
14. P. Brookes and P. D. Lawley, Biochem. J., 80, 496 (1961).
15. T. V. Venkstern and A. A. Baev, Absorption Spectra of Minor Components in Some Oligonucleotides of RNA [in Russian], Vol. 6, Nauka, Moscow (1967).
16. G. H. Beaven, E. R. Holiday, and E. A. Johnson, in: Nucleic Acid Chemistry and Biology, Vol. 1, New York (1955), p. 495.
17. Z. Dische, Methods in Carbohydrate Chemistry, Vol. 1 (1962), p. 481.
18. R. Block, in: Analytical Methods of Protein Chemistry [Russian translation], Inostr. Lit., Moscow (1963), p. 471.
19. A. M. Belikova and N. I. Grineva, Mol. Biol., 4, 697 (1970).
20. M. Tomasz, Biochim. Biophys. Acta, 199, 18 (1970).