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Principles of Selective Inactivation of a Viral Genome. Comparative Kinetic Study of Modification of the Viral RNA and Model Protein with Oligoaziridines

E. A. Tsvetkova and N. M. Nepomnyaschaya*

Zelinskii Institute of Organic Chemistry, Russian Academy of Sciences, Leninskii pr. 47, Moscow, 117913 Russia; fax: (095) 932-9659; E-mail: nmnkro@dialup.ptt.ru

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Abstract—Comparative kinetic analysis of inactivation of bacteriophage MS2 infectivity and aminoalkylation of a model protein (trypsin inhibitor) with oligoaziridines was performed in order to evaluate the selectivity of viral RNA modification with oligocationic reagents. The transition from ethyleneimine monomer to di-, tri-, and tetramer leads to a sharp increase in the rate constant of infectivity inactivation, whereas the rate constant of protein modification changes insignificantly. The selectivity coefficient of the phage RNA aminoalkylation relative to trypsin inhibitor modification increases in this series by more than an order of magnitude. This effect is probably associated with the strengthening of the reagent binding to the nucleic acid, which implies a reaction mechanism that involves the formation of a reactive intermediate. The latter might be an electrostatic complex of the oligocationic reagent and RNA, the only polyanion in the virion. A pronounced decrease in the rate constant of infectivity inactivation in the presence of multiply charged anions (in phosphate buffer) and a biogenic polyamine (spermine) favors this hypothesis. Increasing the reaction temperature increases the rate constant of infectivity inactivation and decreases selectivity of the viral RNA modification.

Key words: infectivity inactivation, selectivity, ethyleneimines, MS2 phage, trypsin inhibitor, viruses

Selective chemical modification of nucleic acids in the presence of other biopolymers is a main requirement in the development and use of many biotechnological processes, in particular when preparing inactivated vaccines and when inactivating viral contaminations in biotechnological preparations [1, 2]. Increase in inactivation selectivity (i.e., increase in the modification rate of nucleic acids compared to that of other virion components) allows inactivation of the virus infectivity to the required depth. Importantly, the effectiveness and specificity of the immune response as well as the structure and function of other biopolymers in the reaction mixture are practically unaffected in this case.

Ethyleneimine and its derivatives are widely used for preparing inactivated antiviral vaccines [3-9]. Alkylation of nucleic acid components with ethyleneimines blocks their template synthesis, which accounts for the virus infectivity inactivation in the presence of these reagents.

It has been previously shown that, upon transition from ethyleneimine monomer to its oligomers (di-, tri-, and tetramers), the inactivation rate of bacteriophage MS2 infectivity sharply increases [10]. An oligoaziridine molecule contains more than one protonizable group and, hence, is an oligocation in solution. It was shown that oligoaziridines protonated on the aziridine nitrogen display the highest reactivity in the aminoalkylation reaction [11]. Upon transition from ethyleneimine monomer to tetramer, the ability for protonation of the aziridine nitrogen decreases. For this reason, the authors of [11] suggested that the sharp increase in the inactivation rate in this reagent series is associated with the root-mean-square concentration of the reagent near the viral genome due to Coulomb affinity of oligocations for polyanions (nucleic acid). An increase in the average positive charge and number of protonizable groups in the reagent molecule should not influence the rate of chemical modification of the virion protein coat and other biopolymers contained in the reaction mixture. Therefore, in this case we expect an increase in the selectivity of infectivity inactivation.

When using oligocationic inactivating reagents, besides the number and relative position of the protonizable groups in the reagent molecule, some other factors that determine the extent of its affinity for nucleic acids should be considered. These are pH, ionic strength, and

* To whom correspondence should be addressed.

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ionic composition of the reaction medium [1]. These parameters directly affect the extent of electrostatic affinity of the oligocation for the polyanion and, therefore, the rate of chemical reaction between them.

Let us assume that the aminoalkylation rate of other biopolymers in the reaction mixture correlates with the content of the reactive (i.e., protonated by the aziridine nitrogen) form of the reagent. Selectivity of the viral RNA modification should then sharply (by several orders of magnitude) increase upon transition from the ethyleneimine monomer to tetramer [10].

The goal of this study was to evaluate the selectivity of oligoaziridines in the reaction with phage RNA with respect to model protein modification as the ratio between the effective rate constants of these processes determined by independent kinetic methods. Comparative kinetic analysis with regard for the peculiarities of interaction of oligocationic reagents with nucleic acids allows for development of novel approaches to reagent selection and conditions of chemical inactivation of viral infectivity in solution.

MATERIALS AND METHODS

Ethyleneimines. Monomer (I), dimer (II), trimer (III), and tetramer (mixture of IV and V) of ethyleneimine (Fig. 1) were obtained in the laboratory of R. G. Kostyanovsky (Institute of Chemical Physics, Russian Academy of Sciences). The purity of the preparations and the composition of the isomeric mixture of tetramers were monitored by proton magnetic resonance by comparing the spectra of freshly distilled preparations with those reported in the literature [12]. Ethyleneimine solutions were prepared immediately before use. The concentrations of solutions prepared by weighing a batch of the reagents were monitored colorimetrically by determining the aziridine group.

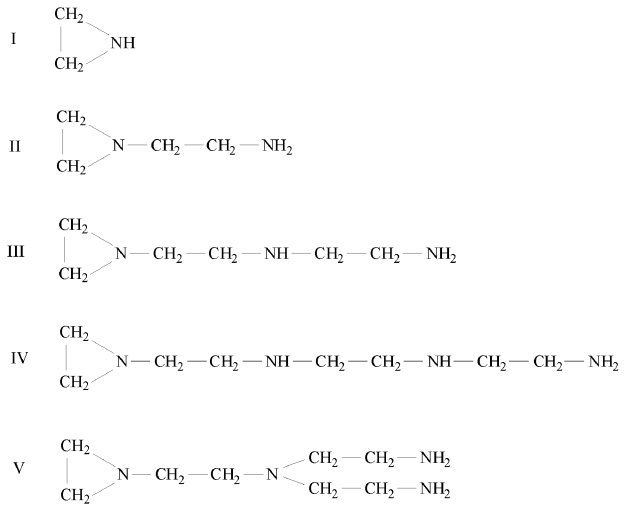

Fig. 1. Ethyleneimine (I) and its oligomers: dimer (II), trimer (III), and tetramers (linear (IV) and branched (V)).

Quantitative determination of aziridines in the solution. Precise concentrations of aziridine-containing reagents in the solution and relative change in the concentration during incubation of the reagent solution were determined colorimetrically by the reaction with 4-(*p*-nitrobenzyl)pyridine (NBP) (Sigma, USA) as described in [13].

Bacteriophage MS2 was accumulated by a conventional procedure as described in [14]. After removal of the cell debris, the phage was purified by double precipitation with polyethylene glycol PEG 6000 (Merck, Germany) [15], then suspended in 0.15 M NaCl and stored at 4°C. The phage infectivity titer was determined by the method of agar bilayer on bactotryptone agar (Difco, USA) using the XL1 (F^+) strain of *E. coli*.

Soybean trypsin inhibitor (Reanal, Hungary) was used without additional purification.

Inactivation of phage MS2. Freshly prepared working solution of the reagent in 0.15 M NaCl was diluted with the reaction buffer to the required concentration (Fig. 2), and the calculated volume of the phage suspension with the original titer of 10^{11} PFU per ml was added. The reaction mixture was then incubated at room temperature or in a thermostat at 37°C. Aliquots of the mixture were taken at certain intervals and immediately diluted 10-100-fold with 0.15 M NaCl. Then serial dilutions were prepared and the infectivity titer of the inactivated suspension was determined. Effective rate constants of infectivity inactivation (k_1) were determined based on the survival curves according to the equation:

$$k_1 = 2.3/ct \log S_0/S_t , \quad (1)$$

where c is the reagent concentration in the solution and S_0 and S_t are the infectivity titers of the phage suspension before inactivation and at time t, respectively.

Standard deviations of the rate constants were calculated from the results of several independent experiments.

Modification of trypsin inhibitor with ethyleneimines. Trypsin inhibitor was incubated with the reagent (at final concentrations of 1 mg/ml and 10 mM, respectively) in 0.2 M MOPS (sodium 3-[N-morpholino]propanesulfonate, Sigma), pH 7.0, at 20 or 37°C. At certain times, aliquots were taken from the reaction mixture and the residual activity of the reagent was blocked by adding 3 volumes of 0.5 M sodium thiosulfate. After dialysis against 0.5% glycerol, the specimens were lyophilized on a SpeedVac rotor concentrator (Savant Instruments, USA). The samples were analyzed by isoelectric focusing (IEF) in a thin gel (110 × 120 × 0.4 mm; T = 7.5%, C = 3%) containing 2% ampholytes (pH 4.0-6.0) (Institute of Chemistry, Academy of Science of Estonia, Tallinn, Estonia). IEF was conducted at constant power (5-6 W) at 10°C for 2 h using 1 M

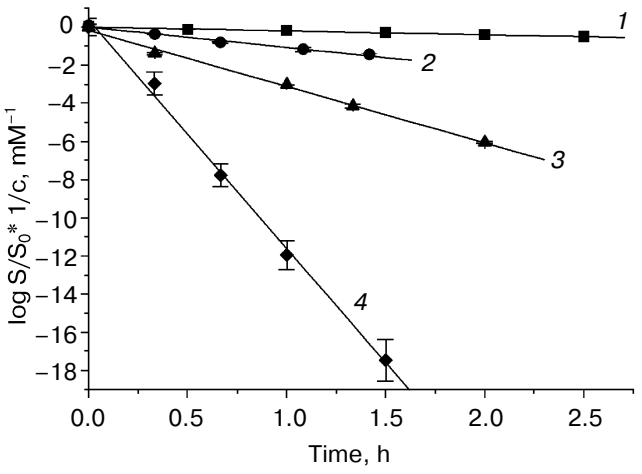

Fig. 2. Survival curves of phage MS2 treated with ethyleneimines in 0.2 M MOPS, pH 7.0, at 20°C: *1*) monomer, 10 mM; *2*) dimer, 3 mM; *3*) trimer, 1 mM; and *4*) tetramer, 0.2 mM.

NaOH and 1 N H_3PO_4 as electrode solutions [16]. After fixing the proteins with trichloroacetic acid, staining with Coomassie R-250 (Serva, Germany) and subsequent destaining of the background, the gel was placed on transparent polymeric film, laminated, and scanned at optical resolution 300 × 600 dpi on a Genius Color Page-CS scanner (KYE Systems Corp., Taiwan). The extent of conversion in the reaction of trypsin inhibitor modification was determined by the decrease in the intensity of the characteristic band corresponding to the original (unmodified) protein. To monitor the protein state during the incubation, a similar experiment was performed in ethyleneimine-free reaction mixture.

Calculation of kinetic characteristics of protein modification. *Determination of the overall rate constant of reagent consumption for side reactions*. At certain intervals (at the beginning, in the middle, and at the end of incubation), aliquots were taken from the reaction medium containing the modified protein, and the residual concentration of aziridine groups was determined by the reaction with NBP (see "Quantitative determination of aziridines in the solution"). The rate constant of consumption of the reagent in side reactions, k', was determined using the equation of first order reaction kinetics. A similar experiment performed in protein-free reaction mixture served as a control.

Determination of the rate constant of protein modification. The rate constant of protein modification, k_2, was calculated by approximating the experimental kinetic curve using the equation:

$$\ln \frac{P_0}{P_t} = \frac{A_0 k_2}{k'} (1 - e^{-k' \cdot t}) , \qquad (2)$$

where A_0 is the initial concentration of the reagent in the solution, P_0 and P_t are the fractions of the characteristic band corresponding to the original protein before reaction and at time t, respectively, and k' is the experimentally determined rate constant of the consumption of the reagent in side reactions.

RESULTS

Figure 2 shows typical survival curves of MS2 bacteriophage after treatment with ethyleneimines.

The exponential character of the decrease in the infectivity titer after treatment of MS2 phage with ethyleneimine and its derivatives indicates that the concentration of the reagent is constant during the inactivation period. The rate constant of infectivity inactivation significantly increases with the number of protonizable groups in the reagent molecule, the most pronounced increment being observed on the transition from ethyleneimine monomer to trimer and, especially, tetramer. Table 1 contains the average values of the effective rate constants of inactivation for the studied reagents.

The increase in the rate constant with increasing the number of ethyleneimine units in the reagent molecule is in good agreement with data obtained before under other inactivation conditions (0.15 M NaCl, pH 7.5) [10].

Typical results of the analysis of trypsin inhibitor by IEF after its treatment with ethyleneimines are shown in Fig. 3. As the duration of the incubation increases, the intensity of the characteristic (lower) band corresponding to the original protein markedly decreases. Conversely, the intensity of the band corresponding to modification products with higher p*I* values increases. Blank experiments performed in reagent-free medium demonstrated stability of the protein over the entire incubation period both at room temperature (Fig. 3e) and at 37°C. For this reason, all changes in IEF characteristics were attributed to protein modification by the ethyleneimines.

Table 1. Rate constants of inactivation of phage MS2 infectivity with ethyleneimine and its oligomers in 0.2 M MOPS, pH 7.0, at 20°C

Reagent	k_1, $M^{-1} \cdot min^{-1}$
Monomer (EI)	8.2 ± 0.8
Dimer (DEI)	42.7 ± 2.4
Trimer (TEI)	123 ± 20
Tetramer (TetrEI)*	472 ± 20

* Hereafter, ethyleneimine tetramer means the mixture of linear and branched isomers.

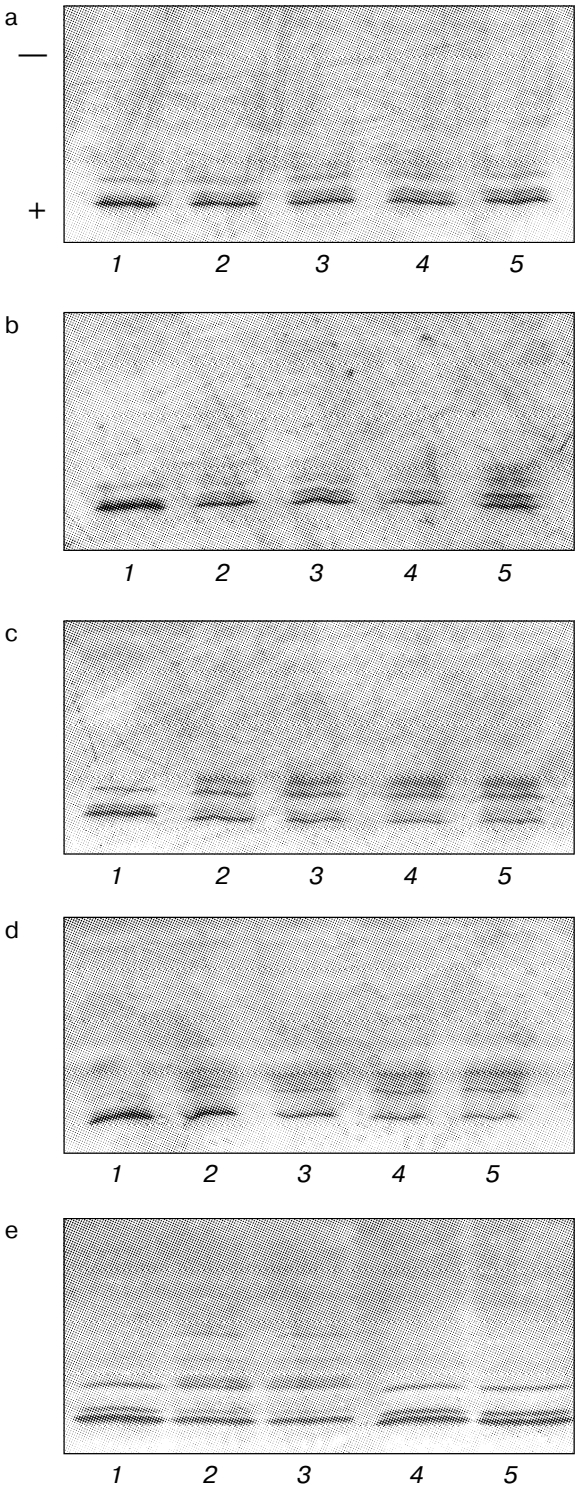

Fig. 3. IEF analysis of trypsin inhibitor after its treatment with 10 mM ethyleneimines in 0.2 M MOPS, pH 7.0 (a-d), and 0.2 M sodium acetate, pH 7.0 (e) at 20°C. The incubation time of the reaction mixture is expressed in hours. a) Monomer: *1*) 0; *2*) 25; *3*) 44.5; *4*) 72; *5*) 96.5; b) dimer: *1*) 0; *2*) 23.5; *3*) 45.5; *4*) 63.5; *5*) 109); c) trimer: *1*) 0; *2*) 22; *3*) 46; *4*) 71; *5*) 94); d) tetramer: *1*) 0; *2*) 21; *3*) 47; *4*) 71; *5*) 87.5; e) trimer: *1*) 0; *2*) 44; *3*) 72; *4*, *5*) control without reagent: *4*) 0; *5*) 72 h.

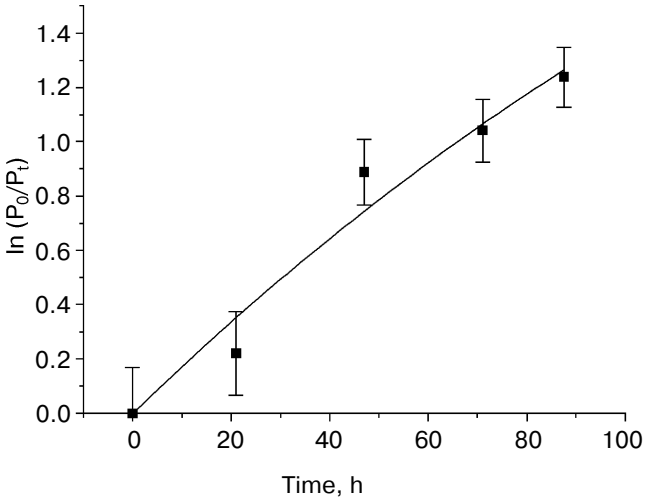

Fig. 4. Kinetic curve of trypsin inhibitor modification with 10 mM ethyleneimine tetramer in 0.2 M MOPS, pH 7.0, at 20°C.

A typical kinetic curve of trypsin inhibitor modification with ethyleneimines that was obtained by quantitative optical analysis of IEF gels is shown in Fig. 4. Given that the ethyleneimine concentration in the reaction mixture is not constant over the whole period of protein treatment, the results were approximated by Eq. (2), which accounts for the consumption of the reagent in side reactions. When approximating, we considered the total rate constant of the reagent consumption k', which was determined independently by a colorimetric procedure, as a fixed parameter. Experimental k' values obtained in the presence and absence of the modified protein did not differ within the experimental error. This finding suggests that consumption of the reagent in the main reaction is negligible. Therefore, the reagent is present in the reaction mixture in excess to the reaction sites of the protein.

Table 2 summarizes the rate constants of trypsin inhibitor modification with ethyleneimines in MOPS at pH 7.0. The data show that activity of the reagents of the oligoaziridine series towards model protein varies insignificantly, showing no pronounced tendency to change with the number of ethyleneimine units in the reagent molecule. In addition, no dependence between the number of protonizable groups and stability of the reagent in the solution was found. For example, ethyleneimine trimer that modifies the protein with maximal (within the measurement error) rate exhibits the lowest stability under our experimental conditions.

We used soybean trypsin inhibitor as a model protein to study modification kinetics with oligoaziridine because of easy interpretation of the results of isoelectric focusing in polyacrylamide gel. The analysis showed that the kinetic data have practically no dependence on the nature of the protein. To confirm this finding, we performed experiments on modification of human serum albumin (HSA) (Sigma, USA) under the same conditions as used for

Table 2. Rate constants of trypsin inhibitor modification with ethyleneimine and its oligomers (k_2) and rate constants of the reagent consumption in side reactions (k') in 0.2 M MOPS, pH 7.0, at 20°C

Reagent	k', min^{-1}	k_2, $M^{-1}\cdot min^{-1}$
Monomer	$(1.5 \pm 0.17) \cdot 10^{-4}$	$(1.2 \pm 0.15) \cdot 10^{-2}$
Dimer	$(0.54 \pm 0.11) \cdot 10^{-4}$	$(1.3 \pm 0.46) \cdot 10^{-2}$
Trimer	$(2.2 \pm 0.17) \cdot 10^{-4}$	$(4.6 \pm 1.10) \cdot 10^{-2}$
Tetramer	$(0.80 \pm 0.08) \cdot 10^{-4}$	$(2.6 \pm 0.41) \cdot 10^{-2}$

Table 3. Comparison of the selectivity of oligoethyleneimines during aminoalkylation of phage MS2 genome and modification of the model protein (s). Relative rate constants of infectivity inactivation (k'_1) and trypsin inhibitor modification (k'_2) in 0.2 M MOPS, pH 7.0, at 20°C (as normalized to the monomer)

Reagent	$k'_1 = k_1/k_{1EI}$	$k'_2 = k_2/k_{2EI}$	$s' = k'_1/k'_2$
Monomer	1.00 ± 0.20	1.00 ± 0.25	1.00 ± 0.45
Dimer	5.19 ± 0.81	1.02 ± 0.50	5.1 ± 3.3
Trimer	14.9 ± 3.90	3.71 ± 1.34	4.0 ± 2.5
Tetramer	57.3 ± 8.12	2.11 ± 0.60	27 ± 11

trypsin inhibitor. Preliminary experiments showed that HSA is not modified with oligoaziridines as rapidly as trypsin inhibitor. However, on the whole, the kinetic characteristics were similar to those obtained for trypsin inhibitor.

Comparative analysis of data presented in Tables 1 and 2 allows direct evaluation of the selectivity of the effect of oligocationic reagents on the virion nucleic component. For the sake of simplicity, we assumed that, in contrast to oligocations, ethyleneimine monomer does not exhibit selectivity towards RNA because of its minimal electrostatic affinity for the polyanion. Therefore, the selectivity (s) of each reagent (i), which is quantitatively expressed as the ratio of rate constants of conventional competitive processes k_1 and k_2, can be normalized relative to the ethyleneimine monomer:

$$s_i = \frac{k_{1i}}{k_{2i}}; \qquad s_{EI} = \frac{k_{1EI}}{k_{2EI}};$$

$$s'_i = \frac{s_i}{s_{EI}} = \frac{k_{1i}/k_{2i}}{k_{1EI}/k_{2EI}} = \frac{k_{1i}/k_{1EI}}{k_{2i}/k_{2EI}} = \frac{k'_{1i}}{k'_{2i}}. \quad (3)$$

The results of comparative analysis of two model processes are summarized in Table 3 and Fig. 5.

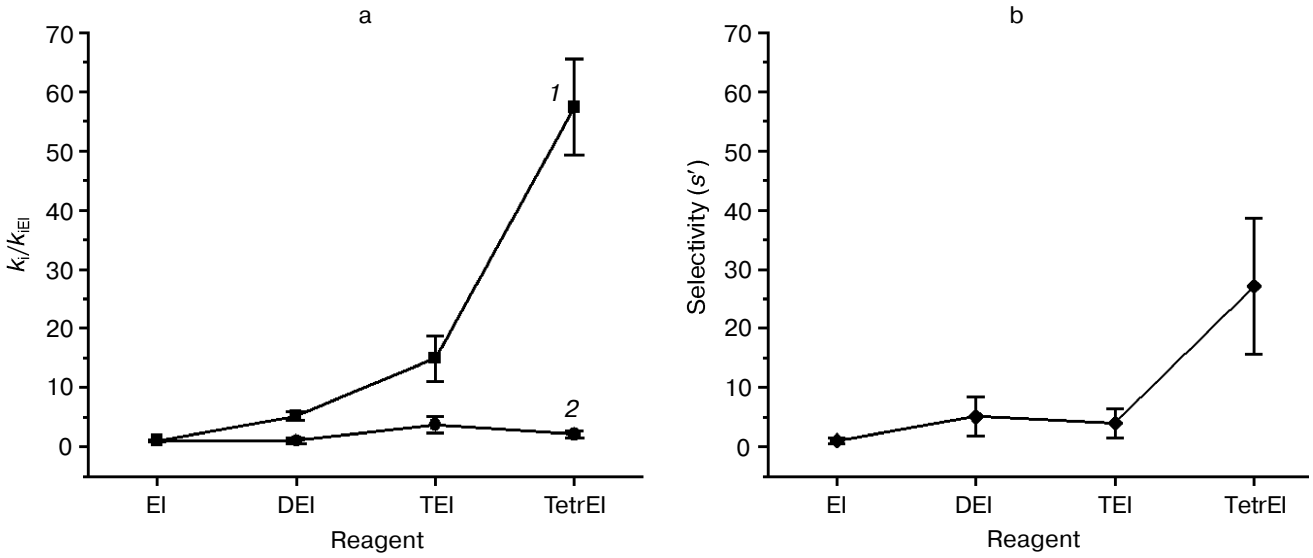

Fig. 5. a) Change in the rate constant (k_1) of inactivation of phage MS2 infectivity (*1*) and rate constant (k_2) of trypsin inhibitor modification (*2*) in the series of oligoethyleneimine reagents in 0.2 M MOPS, pH 7.0, at 20°C. Rate constant values are normalized relative to the ethyleneimine monomer. b) Change in the selectivity of oligoethyleneimines during inactivation of phage MS2 infectivity as compared to trypsin inhibitor modification (0.2 M MOPS, pH 7.0, 20°C) depending on the number of protonated groups in the reagent molecule.

Table 4. Rate constants of inactivation of phage MS2 infectivity (k_1) and trypsin inhibitor modification (k_2) with ethyleneimine trimer in 0.2 M MOPS, pH 7.0, at 20 and 37°C

Reaction temperature	k_1, $M^{-1}\cdot min^{-1}$	k_2, $M^{-1}\cdot min^{-1}$
20°C	123 ± 20	$(4.6 \pm 1.10) \cdot 10^{-2}$
37°C	391 ± 71	$(3.3 \pm 0.40) \cdot 10^{-1}$
Process	$k_{i\,(37°C)}/k_{i\,(20°C)}$	
Infectivity inactivation	3.2 ± 1.1	
Protein modification	7.3 ± 2.6	

The character of changes in the selectivity on transition from ethyleneimine monomer to tetramer generally correlates with the increase in the rate constant of infectivity inactivation because the values of the rate constant of protein modification in this reagent series differ insignificantly.

Next, we analyzed phage MS2 infectivity inactivation and trypsin inhibitor modification at 37°C. The goal of this experiment was to compare the relative increase in the rate constants k_1 and k_2. The results are shown in Table 4. As seen from the table, the temperature coefficient of the model protein modification is higher than that of the phage genome modification, which suggests a decrease in the selectivity of infectivity inactivation on increase in temperature.

Considering the electrostatic nature of the affinity of oligocationic reagents for the polyanionic substrate (RNA), the rate and selectivity of viral genome modification should depend on the nature of the ions contained in the reaction buffer. Table 5 shows the rate constants of inactivation of phage MS2 infectivity determined in different buffers, pH 7.0, at 20°C.

In media where the ionic background is formed by single-charged ions (MOPS, acetate buffer, or sodium chloride), the differences in the values of the rate constant of infectivity inactivation are within the limits of experimental error, i.e., the activity of the reagents varies insignificantly. When phosphate buffer at the same concentration is used, inactivation proceeds much more slowly, the rate constant increasing only in 3.7 times (in contrast to almost 60-fold increase in 0.2 M MOPS) upon the transition from the ethyleneimine monomer to tetramer.

The presence in the solution of multiply charged cations that compete with the reagent for binding with nucleic acids also affects the rate of viral genome modification. Figure 6a illustrates a sharp decrease in the rate of phage MS2 inactivation with 1 mM ethyleneimine trimer in the presence of 3 mM spermine.

Factors that determine the rate of viral infectivity inactivation with oligoethyleneimines and affect the selectivity of this process include pH and ionic strength of the reaction medium. The effect of these factors on the rate of inactivation of phage MS2 infectivity with ethyleneimine trimer is shown in Fig. 6b. On increase in the salt concentration in the solution from 0.15 to 0.5 M, the process is retarded more than 10-fold, and pH increase from 7.0 to 8.0 in the medium with high salt concentration completely stops inactivation.

DISCUSSION

It is known that covalent modification of any nucleotide residue in viral RNA prevents its complete replication, thus terminating virus reproduction [17]. Aziridine-containing reagents attack nucleophilic sites in nucleic bases predominantly in positions N7, N3, and N1 in purines and, to lesser extent, N3 in pyrimidines [18-20]. Imidazole ring opening that occurs due to aminoalkylation with ethyleneimines in position N7 completely blocks template synthesis, the rate of this stage being significantly higher than in the case of other alkylating agents [17, 21]. Therefore, phage MS2 infec-

Table 5. Rate constants of inactivation of phage MS2 infectivity (k_1, $M^{-1}\cdot min^{-1}$) with oligoethyleneimines in different ionic media, pH 7.0, at 20°C

Reagent	0.2 M MOPS	0.2 M acetate buffer	0.15 M NaCl	0.2 M phosphate buffer
Monomer	8.2 ± 0.8			3.3 ± 0.4
Dimer	42.7 ± 2.4			
Trimer	123 ± 20	86.0 ± 16	110 ± 21	10.0 ± 1.0
Tetramer	472 ± 20			12.1 ± 1.6

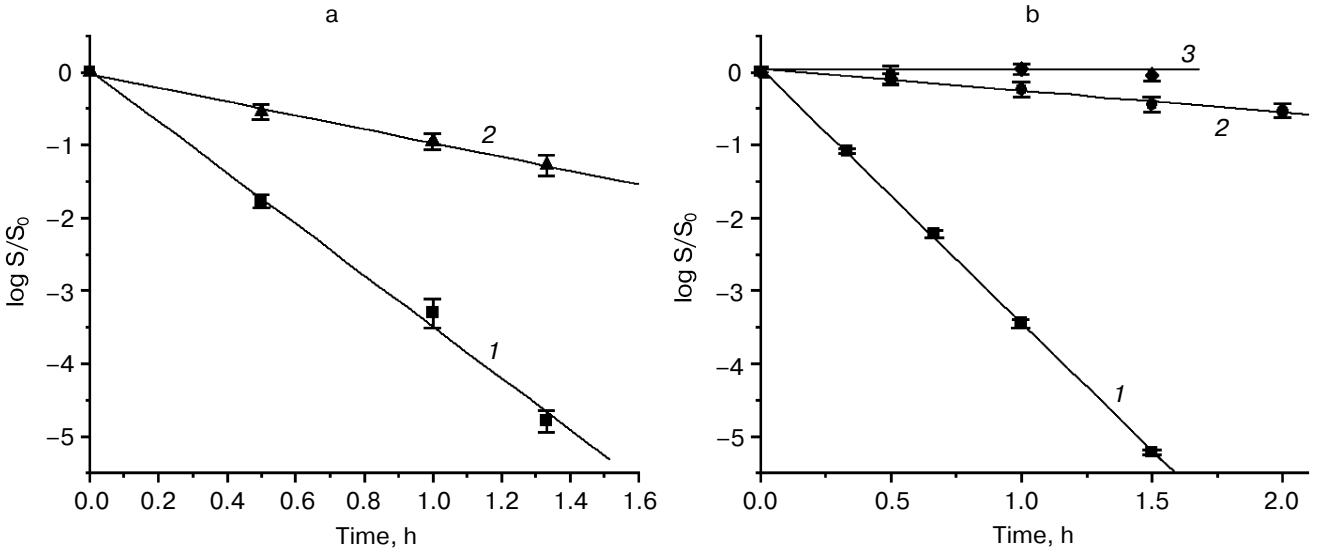

Fig. 6. a) Survival curves for phage MS2 during treatment with 1 mM ethyleneimine trimer in 0.15 M NaCl, pH 7.0, at 20°C, in the absence (*1*) and presence (*2*) of 3 mM spermine; b) survival curves for phage MS2 during treatment with 0.3 mM ethyleneimine trimer in 0.025 M phosphate buffer at 37°C: *1*) in the presence of 0.15 M NaCl, pH 7.0; *2*) in the presence of 0.5 M NaCl, pH 7.0; *3*) in the presence of 0.5 M NaCl, pH 8.0.

tivity is inactivated when the first modified residue in the RNA molecule occurs, and the rate of this process is in a good correlation with the rate of chemical modification of the viral genome.

According to the literature, aminoalkylation of nucleic acids can also involve phosphate residues [22, 23]. In RNA, this process is sometimes associated with breaks of the phosphodiester bonds [24], causing loss of infectivity. However, in this work we did not intend to study the composition and structure of the products of genome modification. Our main goal was to compare integral kinetic characteristics of model virus infectivity inactivation and those of model protein modification as well as to test the assumption that selectivity of the oligocationic reagents towards nucleic acids increases with the number of protonizable groups in the reagent molecule.

Data presented in Tables 1 and 2 indicate that, upon transition from ethyleneimine monomer to tetramer, the rate of inactivation of phage MS2 infectivity increases more that 50-fold. However, the rate of model protein modification does not change more than 10-fold and without regard to the number of the ethyleneimine units in the reagent molecule. This finding is consistent with the hypothesis that oligocationic reagents display electrostatic affinity for nucleic acid, a representative polyanion. This affinity will most likely result in a sharp increase of the reagent concentration near the reaction sites in RNA compared to the average concentration of the reagent in the solution. Thus, the dependence of the selectivity on the number of cationogenic groups in the reagent molecule is determined mainly by the character of the change in the rate constant of phage genome modification (Fig. 5).

In our previous work [10], we assumed that the reactive reagent form is protonated on the aziridine nitrogen. In accordance with pK_a values for the aziridine group, the content of the protonated form of aziridine in the solution at a specified pH value should sharply decrease upon transition from ethyleneimine monomer to tetramer (by approximately four orders of magnitude at pH 7.0). With regard for more than 50-fold acceleration of infectivity inactivation in the series, it is obvious that the reagent state in the vicinity of RNA is qualitatively different from the free reagent state in the solution. This can be due to preliminary binding of the reagent by the polyanionic substrate, changing the reaction mechanism from bimolecular (in the case of ethyleneimine monomer) to a more complex one that involves reactive intermediate formation (in the case of oligomers).

The structure of oligoethyleneimine molecules implies the presence of two functionally different sites, i.e., the substrate binding site (whose function is fulfilled by the ethyleneimine units) and the reactive site (i.e., the aziridine group). On one hand, formation of the intermediate should decrease the activation energy of the interaction between the reactive sites of the substrate and the reagent. On the other hand, it should increase the aziridine group activity during the attack of nucleophilic sites due to protonation near the negative charge of the nucleic acid. Strictly speaking, the reaction of aminoalkylation of the viral genome in this case should be described in terms of stationary kinetics. However, the method of

infectivity inactivation does not allow work over a sufficiently broad concentration range of the reacting compounds suitable for determination of the parameters of an enzyme-like reaction. In addition, we can only speak of the change in the ethyleneimine selectivity towards RNA upon transition from ethyleneimine monomer to its oligomers in the frame of a unified formal kinetic scheme. In view of this, for the sake of simplicity, we described the process of nucleic acid modification in terms of pseudo-first order reaction kinetics.

The second main prerequisite for the hypothesis of a sharp (by several orders) increase in selectivity towards polynucleotides in the series of oligoethyleneimines [1, 10] was the assumption that the mechanism of modification of other components of the virion (its protein coat, in particular) remains bimolecular because of the absence of specific affinity for the reagent. Therefore, the rate of aminoalkylation of these components should be determined only by the concentration of the protonated aziridine form in the solution, i.e., it should decrease by many times upon transition to higher oligomers in accordance with the pK_a values. However, the direct kinetic study of model protein modification refuted this assumption. As seen from Table 2, the activity of higher ethyleneimine oligomers towards trypsin inhibitor not only is not lower, but is even higher than that of monomer. It is most likely that an elementary interaction between the protein and oligoethyleneimine is not a simple bimolecular co-hit as could be assumed for a standard modifier that does not exhibit specific affinity for the protein.

In this work, we did not intend to study the mechanism of model protein modification with oligoaziridines. However, the assumption can be made that the absence of correlation between the rate of the process and the content of the protonated form of aziridine in the solution is due to partial binding of the reagent by the protein. As a result, the electrophilic properties of the aziridine group cease to depend on the total charge of ethyleneimine molecule. In addition, it favors this hypothesis that trypsin inhibitor at neutral pH values is an anionic protein ($pI = 4.5$) and, hence, contains regions with higher density of negative charge, which should display affinity for oligocations.

Of all possible reactions between aziridines and proteins, the reaction with cysteine is best studied [25]. Soybean trypsin inhibitor consists of a single peptide chain with two disulfide bridges and does not contain free SH-groups [26]. However, site for nucleophilic attack of the aziridine group displaying sufficiently high electrophilicity may include nucleophilic sites of other amino acid residues (i.e., histidine, lysine, tryptophan, and aspartic and glutamic acids).

Thus, we suggest that the main and only reason for the increase in selectivity towards viral polynucleotide, which is observed upon the transition from ethyleneimine monomer to tetramer, is the increase in the effectiveness of electrostatic binding of oligoaziridines by the nucleic acid with the formation of a reactive intermediate. Table 3 shows that, upon the transition from ethyleneimine monomer to tetramer, the selectivity increases approximately 30-fold, rather than several orders of magnitude, as assumed earlier based on the calculation of the content of the protonated form of aziridine reagents in the solution. Nevertheless, for practical purposes, even such elevation of the selectivity can be considered an important argument favoring the use of oligocationic inhibitors of viral infectivity.

Considering the electrostatic nature of the interaction of oligoethyleneimines with nucleic acids, it could be concluded that the selectivity of infectivity inactivation should be determined by the same factors as the rate of viral genome modification. However, when choosing the optimal conditions for treatment of the inactivated suspension, it should be taken into account that selectivity is expressed by the ratio between the rate constants of competing reactions. For instance, increasing temperature from 20 to 37°C entails a 3-fold increase in the rate constant of phage infectivity inactivation, whereas the rate constant of model protein modification increases 7-fold (Table 4). As a result, a more than twofold decrease in selectivity is observed along with acceleration of the main process.

Among the factors that affect infectivity inactivation rate are pH, ionic strength, and ionic composition of the reaction medium. These characteristics determine the effectiveness of electrostatic interaction of the reagent and polynucleotide, which favors the hypothesis of oligonucleotide binding to form a reactive intermediate.

On increasing pH, the total positive charge of the reagent molecule decreases, thus attenuating its affinity for the nucleic acid. The content of the form protonated on the aziridine nitrogen, which represents the electrophilic site in aminoalkylation reactions, also decreases. For example, increasing pH from 6.5 to 8.5 results in a 60-fold drop in ethyleneimine dimer activity, and this correlates with the decrease in the concentration of its electrophilic form in the solution [10]. For higher oligoethyleneimines, such a simplified interpretation of the dependence of activity on pH would be incorrect because the kinetics of the process is determined by the concentration of the reactive intermediate. The latter depends on the total charge of the reagent molecule and protonation constant of the aziridine group in the reactive complex. The protonation constant can increase in the field of RNA charge, as occurs in enzyme-like pH-dependent reactions. Comparative analysis of the data presented in Table 5 and previously obtained results is indicative of a symbatic mode of the reagent activity change upon transition from ethyleneimine monomer to tetramer for pH 7.0 and 7.5. However, these data are insufficient to make a conclusion about the effect of pH on inactivation selectivity. For this reason, we only note the considerable (more than 3-fold) acceleration of the reaction as pH is decreased from 7.5 to 7.0.

When phosphate buffer is used as the reaction medium, the inactivation rate markedly decreases for all reagents compared to data obtained in MOPS at the same pH value (Table 5). When phage inactivation with ethyleneimine trimer is studied in media where ionic background is comprised of single-charged ions (MOPS, acetate buffer, or NaCl), similar values of the rate constants are obtained. However, when phosphate buffer (containing multiply charged anions) is used, the reaction is slowed by an order of magnitude. This phenomenon may be accounted for by partial binding of the oligocationic reagent by point multiply charged counterions, which decreases the concentration of the reactive intermediate. Comparison of inactivation rate constants upon transition from ethyleneimine monomer to tetramer in different media suggests that the selectivity of infectivity inactivation in phosphate buffer is also considerably lower than in MOPS. For example, the reaction rate in the reagent series in MOPS increases almost 60-fold, whereas in phosphate buffer the ethyleneimine tetramer is only 3.7-fold more active than the monomer. Because selectivity of oligocationic reagents can be determined as an index of their affinity for the nucleic acid, this finding is also consistent with the hypothesis of intermediate binding of the reagent in an electrostatic reactive complex.

When spermine, a natural oligocation that exhibits affinity for nucleic acids, is contained in the reaction medium, the inactivation rate is also sharply slowed down (Fig. 6a). This is, probably, indicative of a decrease in the reactive complex concentration due to the competitive binding of spermine. In this case, spermine should affect both the rate and selectivity of inactivation.

An increase in the concentration of small counterions is another factor that attenuates the binding of oligocationic reagents with nucleic acids as a result of screening their charges. For example, increasing NaCl concentration from 0.15 to 0.5 M slowed phage inactivation by more than an order of magnitude (Fig. 6b). The effect of ionic strength and ionic composition of the medium on the rate of protein modification with ethyleneimines was not studied. However, it can be assumed with high probability that the rate and selectivity of the process in this case change symbatically, because these factors directly determine the efficiency of the reagent binding by the genome.

The study of the factors that affect the infectivity inactivation rate allows several practical conclusions regarding selection of optimal conditions for work with oligocationic reagents to be made. First, the reagent used for viral infectivity inactivation should be chemically stable under the conditions of the experiment. Only in this case can a correct description of inactivation kinetics, which is required for determination of the necessary and sufficient dose of the inactivating reagent (concentration of the reagent and time of viral suspension treatment), be made [1]. In view of this, the use of oligoethyleneimines is associated with experimental difficulties because of their ability to polymerization during long storage, contact with air, pH decrease, or when entering reaction media containing inducers of cationic polymerization. Strictly speaking, reproducible results can be obtained only with oligomers that were freshly distilled under standard conditions. At the same time, they obviously offer considerable promise because of their oligocationic nature. For this reason, optimal experimental procedures for work with these oligomers should be developed.

The advantage of the use of oligoaziridines versus conventional reagents used for chemical and photochemical infectivity inactivation (e.g., formaldehyde, β-propiolactone, dimethyl sulfate, diepoxybutane, UV- and ionizing irradiation) is associated both with the rate of viral genome modification and selectivity of the process that ensures minimal lesion of the virion protein coat and other polymers contained in the treated suspension. Under various experimental conditions, the rate and selectivity of modification can change both symbatically and antibatically. Therefore, the selection of the reagent and conditions for a particular procedure is a complex multifactorial task. It should be noted that quantitative evaluations reported in this work are referred only to the selected model system and reflect only general regularities associated with the increase in the number of cationogenic groups in the reagent molecule. The kinetic characteristics of the process and selectivity of the reagent in the system apparently depend on the virion structure and the nature of protein components. Therefore, each system requires preliminary kinetic experiments to select optimal dose of the reagent and treatment conditions. Nevertheless, the affinity of oligoaziridines for polynucleotides allows for the use of significantly lower concentrations and duration of treatment of the inactivated suspension compared to other reagents, including ethyleneimine. Higher selectivity of oligoaziridines towards nucleic acids decreases by many times the risk of chemical modification of functionally important regions of the protein coat and other biopolymers contained in the reaction medium.

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