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Virus inactivation and protein modifications by ethyleneimines

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Abstract

Virus inactivation by ethyleneimines was first introduced more than 30 years ago. Selective targeting of nucleic acids was reported for oligomeric ethyleneimines. In this study, trimeric ethyleneimine (TEI) was used to inactivate minute virus of mice (MVM; Parvoviridae) and Semliki forest virus (SFV; Togaviridae). The pH-dependency of the inactivation kinetics observed with MVM was different compared to the kinetics reported for other viruses. The higher inactivation rate at higher pH favoured the idea of a mechanism involving protein modifications. Alteration of the isoelectric point and changes in mass could be observed after treatment of soluble proteins with TEI. The uptake of MVM by host cells was reduced or completely blocked by TEI treatment, as shown by monitoring viral internalisation of DNA into target cells. The observed loss of virus infectivity coincided with the inhibition of virus uptake. Thus, virus inactivation by TEI is most likely also a result of chemical modifications of viral surface proteins.

Keywords: Virus inactivation; Ethyleneimine; Aziridine; Protein modification; Semliki forest virus; Minute virus of mice

1. Introduction

An important field in virus research deals with the development of methods for the inactivation of viruses in biological fluids. Many viral inactivation methods target the structural proteins of viruses. The crucial issue in the development of methods for the inactivation of viruses in biological systems is to selectively target the viruses without damaging proteins of interest in these biological preparations.

There are many new inactivation methods under investigation (Suomela, 1993; Ben-Hur and Horowitz, 1996; Roberts, 1996), e.g. treatment of viruses with photosensitisers, oxygen and light, which is mainly based on modification of viral proteins by singlet oxygen (Käsermann and Kempf, 1997, 1998). To overcome the problem of protein modifications, another viral component can be targeted: nucleic acids. The modification of viral nucleic acids will lead to the inactivation of

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the pathogen. In a biological preparation such as stable blood products or recombinant proteins, nucleic acids do not normally represent the compounds of interest. Therefore, the most promising inactivation methods will be those which target the nucleic acids without damaging the proteins. To achieve this goal, one of the known virus inactivating agents, aziridine, was modified to increase its selectivity towards nucleic acids.

It has been known for decades that ethyleneimines, like the monomeric aziridine, inactivate viruses (Warrington et al., 1973; Bahnemann, 1976). Aziridine and acetylethylethyleneimine were proposed for use in the production of killed antiviral vaccines (Bahnemann, 1975). The difference in reactivity towards polynucleotides and proteins of such electrophilic compounds is not very high. Therefore, the selectivity for the inactivation of the viral genome is not very high too.

To improve this selectivity, one method was based on the specific affinity of the inactivating agent for nucleic acids. Hence, the electrophilic compound aziridine was modified to increase its selectivity. The obtained oligomeric compounds with more than one protonizable amino group are potential candidates to act as polycations showing improved affinity towards polyanions such as nucleic acids.

It was claimed that the oligomeric ethyleneimines primarily react with nucleic acids. In accordance with this, pH-dependent inactivation kinetics were observed. The RNA-containing phage MS2 was inactivated by the action of oligomeric ethyleneimines more efficiently in slightly acidic conditions than in an alkaline environment (Budowsky et al., 1996; Budowsky, 1997), and the higher efficiency of inactivation at more acidic pH was postulated to be due to a higher fraction of the reactive form of the aziridino group at lower pH. In further studies, similar effects were reported for enveloped and non-enveloped viruses (Budowsky, 1998). However, electrophilic compounds like monomeric and trimeric ethyleneimines (TEIs) potentially react with nucleophiles. Therefore, a modification of nucleophilic protein residues (e.g. thioles, amines, alcohols and phenols) should be considered.

In this report, we have studied the mechanism of virus inactivation mediated by ethyleneimines (aziridine and its trimeric form). In particular, the work was focused on the question of whether proteins were also modified and how virus penetration and uncoating were affected during the inactivation procedure.

2. Materials and methods

2.1. Cells and media

Aedes albopictus cells, clone C6/36 (Igarashi, 1978), were grown at 28 °C in Mitsuhashi-Maramorosch medium (Amimed, Switzerland), supplemented with 16% foetal calf serum (FCS), 100 μg streptomycin and 100 U penicillin per ml. Cells were passaged by 1:20 dilution.

Vero cells were grown at 37 °C in medium 199 Hanks (Biochrom, Switzerland), containing 10% FCS, 20 mM HEPES, 100 μg streptomycin and 100 U penicillin per ml. The cells were passaged by 1:20 dilution.

A9-fibroblasts were grown at 37 °C in Dulbecco's modified Eagle's medium (Sigma, Switzerland) in 10% CO_2, containing 10% FCS, 100 μg streptomycin and 100 U penicillin per ml. The cells were passaged by 1:20 dilution.

2.2. Virus propagation

Semliki forest virus (SFV; *Togaviridae*) was propagated in Aedes cells. Briefly, cells were infected with ≈ 10 $TCID_{50}$ (50% tissue culture infectious dose) per cell. At 24 h post infection, the medium was harvested and cellular debris removed by centrifugation (600 g, 10 min). Minute virus of mice (MVM; *Parvoviridae*) was propagated in A9-cells. Cells were infected at a confluency of $\approx 10\%$ with 0.05 $TCID_{50}$ per cell. When the cell monolayer was destroyed, the supernatant was harvested and cellular debris removed. The virus-containing supernatant was aliquoted and stored at -80 °C.

2.3. Synthesis of ethyleneimine

Monomeric ethyleneimine (aziridine) was prepared according to Bestian (Fig. 1(A)) (Bestian, 1963). Briefly, a suspension of 2-aminoethyl hydrogen sulphate (0.25 mol) was slowly added to 50 ml of a boiling solution of sodium hydroxide (20%). Twenty millilitres NaOH (33%) was added simultaneously to the reaction vessel. The desired product was continuously removed from the reaction mixture by fractional distillation. The product was identified by ^{1}H-NMR.

The TEI, *N*-[2-(1-aziridinyl)-ethyl]-1,2-ethanediamine was prepared by acid-induced polymerisation (Fig. 1(B)) (Gembitskii et al., 1972). The desired product TEI was isolated from the crude mixture of oligomeric ethyleneimines by fractional distillation (1–2 mm Hg; 50–60 °C), and its structure was confirmed by ^{1}H- and ^{13}C-NMR spectroscopy. Pure TEI was stored at −80 °C, stock solutions (0.6 M in water) at 4 °C, respectively.

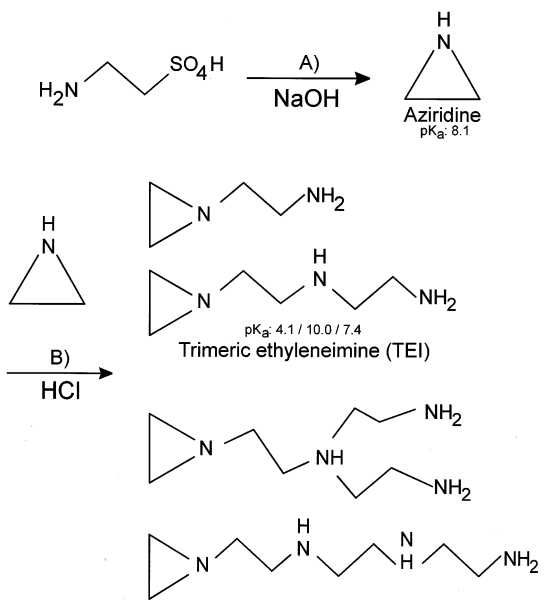

Fig. 1. The reaction scheme for the synthesis of monomeric and trimeric ethyleneimine. (A) Addition of 2-aminoethyl hydrogen sulphate to a boiling sodium hydroxide solution leads to the formation of ethyleneimine (aziridine). (B) Acid-induced polymerisation of aziridine produces different oligomeric ethyleneimines. The trimeric compound *N*-[2-(1-aziridinyl)-ethyl]-1,2-ethanediamine (TEI) was isolated by fractional distillation.

2.4. Inactivation assay

Stock virus in medium was diluted with buffer (MES/MOPS, 0.1 M each) to obtain an initial virus titre of $\approx 10^{9.5}$ $TCID_{50}$ per ml for SFV and 10^8 $TCID_{50}$ per ml for MVM. The respective pH was adjusted prior to incubation and monitored during the incubation: the pH did not vary more than ± 0.1 pH unit from the indicated value during the incubation. To determine the kinetics of inactivation, samples were taken after different incubation times. Ethyleneimine was quenched by the addition of $Na_2S_2O_3$ to a final concentration of 25 mM and residual virus determined by end-point titration.

To determine virus titres, either confluent vero cells (for SFV) or A9-cells grown to 10% confluency (MVM) in 96-well tissue culture plates (TPP, Switzerland) were infected with 50 μl aliquots of 1 in 10 (SFV), or 1 in 5 serial dilutions (MVM) of virus samples (8 wells per dilution). After incubation for 4–7 days (SFV), or 12–15 days (MVM), cytopathic effects (cell destruction) were visualised by staining the remaining viable cells with crystal violet (0.5% in methanol; Fluka, Switzerland). Virus titres were calculated according to the method of Spearman and Kärber (Cavalli-Sforza, 1974) and are indicated as $TCID_{50}$. The inactivation is expressed in reduction factors (RF):

$$RF = \log_{10}\frac{\text{initial virus titre}}{\text{virus titre after inactivation}}.$$

2.5. Protein modifications

Test proteins (myoglobin, ovalbumin, BSA; 150 μM) were incubated in the presence of ethyleneimine (20 mM) at different pH values (6–8) for 24 h at room temperature. In control samples, ethyleneimine was quenched prior to incubation. To test for changes in the isoelectric point of the proteins, samples were analysed by isoelectric focusing using a PhastGel IEF 3–9 on a Phastsystem (Amersham Pharmacia, Switzerland).

To test for modifications by ethyleneimines, myoglobin samples were analysed by electrospray ionisation mass spectrometry (VG Platform mass spectrometer, Micromass Instruments, UK). Raw data were collected between 600 and 1800 mass/charge and processed by the maximal entropy method (MassLynx software). The resolution of the mass spectrometer is $\leqslant 0.01\%$. All samples were adjusted first to pH 7 and further sample preparation was performed using reversed phase desalting tips (C_{18}-ZipTip; Millipore) according to manufacturer's protocol.

2.6. Virus uptake assay

To ensure virus binding, A9-cells were exposed on ice to virus samples that were inactivated before with TEI. For virus uptake, the temperature was raised to 37 °C for different time intervals. Then, to remove external virus, cells were treated with proteinase K and washed at 0 °C. Cells were lysed and internalised viral DNA was detected by real-time PCR (5′ primer: GCACAAGCAGTTGGCAATGT; 3′ primer: CTGGTCTTTCTTCGCAGCCT, amplifying a 265 bp fragment in the NS1 gene of MVM).

3. Results

The monomeric compound ethyleneimine (aziridine) was either prepared according to Fig. 1(A) (Bestian, 1963) or purchased commercially (Serva, Germany). Acid-induced polymerisation of aziridine and subsequent fractional distillation led to pure TEI. The spectral parameters obtained by ^{1}H- and ^{13}C-NMR spectroscopy were in very good accordance with published data (Kostyanovskii et al., 1989). TEI showed no changes in its ^{1}H-NMR spectrum after storing for 10 months at -80 °C.

Fig. 2 illustrates the inactivation kinetics for two different viruses when treated with aziridine and TEI (10 mM) at room temperature. As shown in panels A and B, the enveloped Semliki forest virus (SFV; Togaviridae) was inactivated with aziridine or TEI with no significant differences between acidic and basic conditions (pH 6.5–8.5). The only difference that could be observed was a slightly faster inactivation by TEI (Fig. 2(B)) compared to aziridine (Fig. 2(A)). The non-enveloped minute virus of mice (MVM; Parvoviridae) showed a different behaviour during the inactivation procedure: when exposed to aziridine no significant differences in the kinetics from pH 6–8 could be observed (Fig. 2C). However, when MVM was treated with the TEI, a difference in the kinetics was detected (Fig. 2(D)): at pH 6 (squares) MVM remained almost completely infectious. Upon raising the pH to 7 (circles) or 8 (triangles) an increase in the inactivation capacity of TEI was observed. The inactivation with the monomer was more potent than that with TEI. In all studies, the viruses showed no non-specific loss of infectivity, as determined in control experiments where the virus was incubated under the same conditions without ethyleneimine (stars). In a further set of experiments, the non-enveloped bovine enterovirus (BEV; Picornaviridae), showed no significant loss in infectivity when incubated with TEI at different pH (6–8) up to 24 h at room temperature, as monitored by $TCID_{50}$ on bovine turbinate cells (data not shown).

The surprising observation that MVM lost its infectivity more efficiently at higher pH suggested that not only nucleic acids were involved in the inactivation mechanism, but that proteins were also modified during the procedure. Therefore, we tested whether a reaction with proteins is involved in the inactivation mechanism. Ethyleneimines were incubated with different soluble proteins and subsequent modifications monitored. Modifications of the isoelectric points were detected by isoelectric focusing, as shown in Fig. 3. In the centre of the gels, marker proteins were run (M). Left from the marker, samples of negative controls ($-$) for different pH conditions were run, where TEI was quenched prior to the incubation period. The lanes right from the marker were loaded with samples exposed to TEI ($+$). All three proteins show significant changes in the focusing pattern compared to the negative controls. BSA showed a shift towards higher pH and a less pronounced focusing of the bands (smear) was observed (panel A). Ovalbumin (B) and myoglobin (C) showed additional bands at higher pH

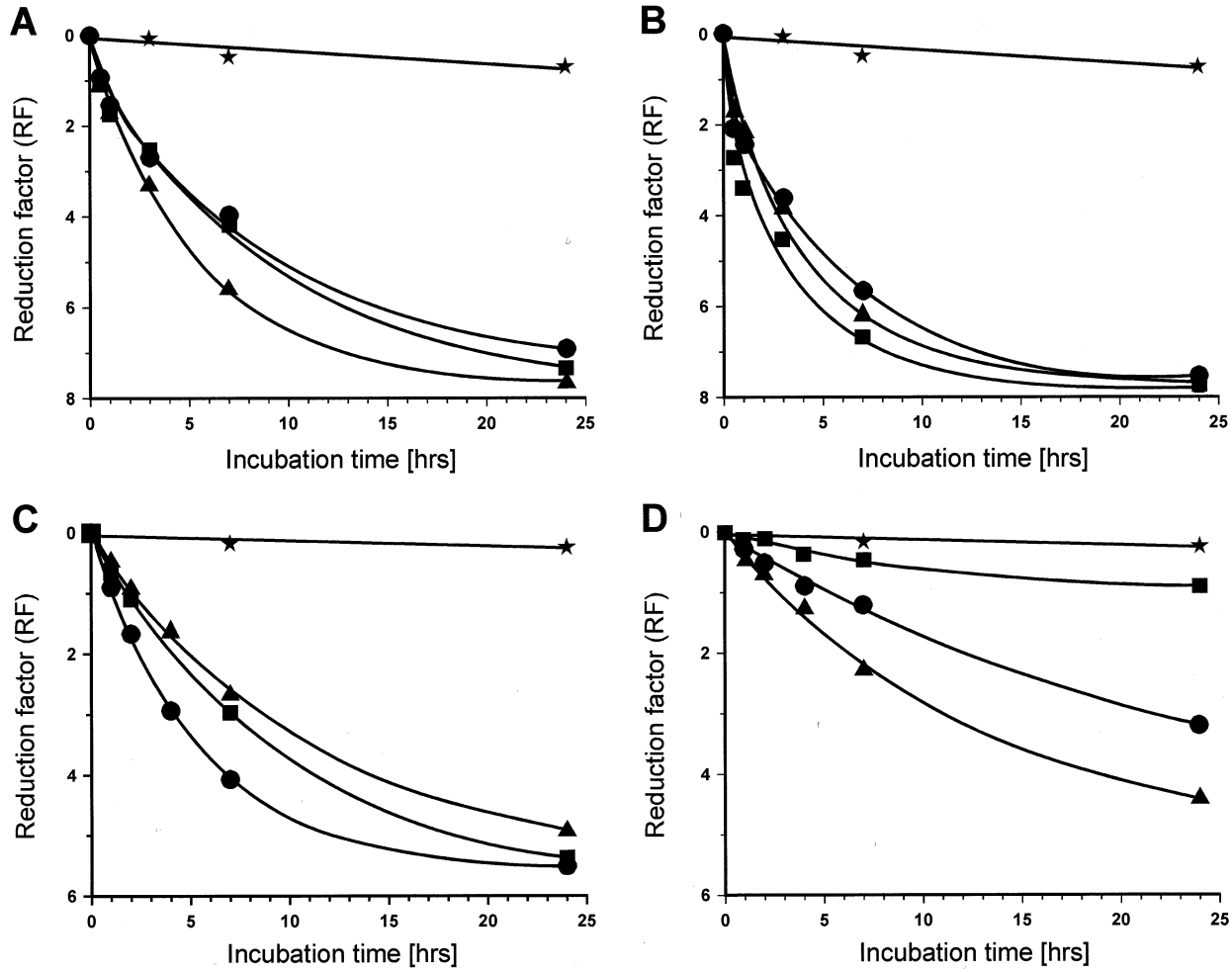

Fig. 2. Kinetics of the inactivation of SFV and MVM by monomeric and trimeric ethyleneimine. Virus was incubated at room temperature with 10 mM ethyleneimines at different pH. After incubation, the ethyleneimines were quenched by the addition of $Na_2S_2O_3$. Virus titres were determined as $TCID_{50}$/ml and inactivation is expressed in reduction factors (log (initial titre/end titre)). Mean values obtained in three independent experiments are shown. Standard errors of means are in the range of 0.5–1 log $TCID_{50}$/ml. (A) SFV was incubated with aziridine at pH 6.5 (■), 7.5 (●) and 8.5 (▲). (B) SFV was incubated with TEI at pH 6.5 (■), 7.5 (●) and 8.5 (▲). (C) MVM was incubated with aziridine at pH 6 (■), 7 (●) and 8 (▲). (D) MVM was incubated with TEI at pH 6 (■), 7 (●) and 8 (▲). In control experiments, virus was incubated without ethyleneimine the mean values from experiments performed at the three indicated pH values are shown (★).

that were badly focused (smear), whilst other bands were less pronounced. A modification of the proteins during exposure to TEI was clearly detectable in all three samples on treatment at pH 6–8.

The modification of myoglobin by ethyleneimine was further analysed by mass spectrometry (Fig. 4). Spectra from probes treated with TEI at pH 6–8 are shown (left panels, from bottom to top). In all experiments, a significant difference from the spectra acquired with the corresponding negative probes, where TEI was quenched prior to incubation (right panels), was observed. In addition to the myoglobin peak at a calculated mass of 16 950 Da, a sequence of several additional peaks with a mass difference

corresponding very well to the mass of TEI (129 Da) can be seen. All spectra were normalised to the half-height of the myoglobin peak. The modification of myoglobin was more pronounced at pH 7 and 8 compared to still detectable changes in mass at pH 6. In further control experiments, where myoglobin was identically treated as the positive probe, but incubated at lower temperature (0 °C), no peaks other than the myoglobin peak could be detected (data not shown).

These results show that in a pH range suitable for virus inactivation protein modification could always be observed. Thus, the loss in infectivity could occur via an inactivation mechanism that involves modification of viral structural proteins. This hypothesis was tested by determining whether virus penetration and uncoating was affected during the inactivation procedure. In a virus uptake assay, the MVM DNA internalised by host cells (A9-cells) was detected by real-time

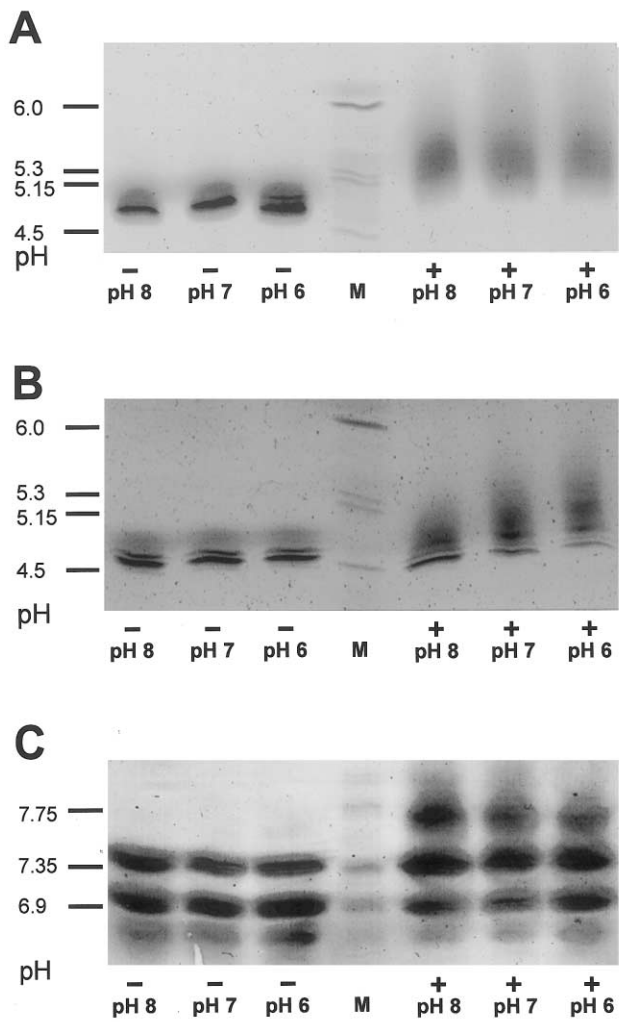

Fig. 3. Modification of the isoelectric point by trimeric ethyleneimine. Solutions of various proteins (150 μM) were incubated for 24 h at room temperature in the presence of 20 mM TEI at pH 6–8. After incubation, the oligomeric ethyleneimine was quenched by the addition of 25 mM $Na_2S_2O_3$ (+). In control experiments, thiosulphate was added prior to the addition of TEI (−). A scanned IEP-PhastGel (pH 3–9) after Coomassie staining is shown. (A) BSA; (B) ovalbumin and (C) myoglobin.

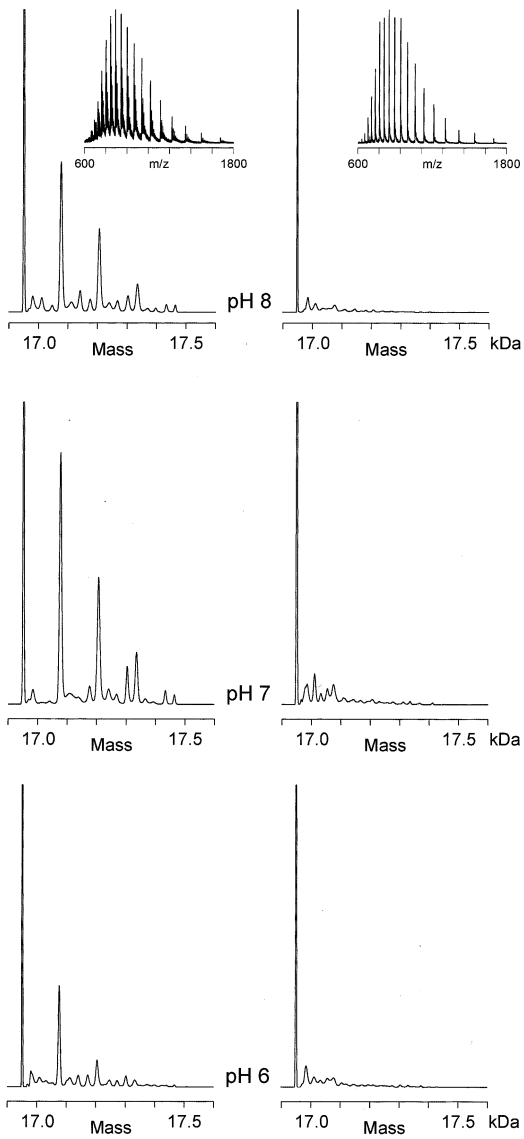

Fig. 4. Analysis of TEI-treated myoglobin solutions by mass spectrometry. The myoglobin solutions described in Fig. 3 were analysed for modifications by mass spectrometry. Ten microlitres of each probe were prepared using a reversed phase desalting tip (C_{18}-ZipTip) and analysed by electrospray ionisation mass spectrometry. In the left panels, mass spectra of samples incubated with TEI at indicated pH (6–8) are shown. In the right panels, mass spectra of the corresponding negative controls (TEI quenched at $t = 0$) are shown. All spectra are normalised to the half height of the myoglobin peak at 16 950 Da. Inset: raw data (shown only for samples incubated at pH 8).

PCR. Virus samples were treated with TEI at different pH values (6–8) for 24 h at room temperature and excessive ethyleneimine was subsequently quenched by the addition of thiosulphate. In Fig. 5 initial virus uptake, detected as internalised DNA and expressed in fluorescence intensity after 50 PCR cycles, is shown. A decrease in virus uptake from the untreated stock virus sample down to the background sample (no virus) was observed in samples treated with TEI at pH 6–8. In negative controls, where ethyleneimine was quenched prior to virus exposure, no decrease in virus uptake compared to the positive control (stock virus) was detected. The addition of 1 mg/ml BSA prior to virus inactivation did not significantly alter prevention of virus uptake (BSA).

When TEI-treated SFV samples were analysed by mass spectrometry, different protein patterns in the mass region of the SFV envelope proteins could be observed compared to negative probes (TEI quenched prior to TEI-incubation or incubation with TEI at 0 °C) (data not shown). These observations support the idea that proteins are involved in the inactivation mechanism.

4. Discussion

Inactivation of virus infectivity by ethyleneimine was predicted to take place mainly via destruction of nucleic acids without concomitant damage to proteins. Obviously such a method, which would not interact with the desired proteins and their activity in biological fluids, would be of great potential for future applications.

The reaction of ethyleneimine was shown to be pH-dependent. The introduction of additional positive charges on the reactive molecule led to an increase in its affinity for polyanions and thus for nucleic acids: the desired target components of viruses. Ethyleneimines are electrophilic agents, and therefore, a virus inactivation mechanism that includes modification of proteins is also possible and was reported for the monomeric compound aziridine (Budowsky, 1998). The reactivity of ethyleneimines is increased by protonation of the

aziridine nitrogen, but is only slightly affected by alkylation. It is, therefore, assumed that the protonated form is the only reactive form for these compounds (Budowsky et al., 1996). When lowering the pH from 8 to 6, the fraction of the reactive form of TEI increased. The inactivation kinetics observed with the RNA phage MS2 confirmed this proposal (Budowsky et al., 1996). In the same manner, other viruses–enveloped (vesicular stomatitis and influenza A virus) and non-enveloped picornaviruses (hepatitis A and foot-and-mouth disease virus)–were reported to be inactivated by oligomeric ethyleneimines faster at pH 7 than at pH 8 (Budowsky, 1998). In contrast to these reports, we could not observe a significant loss of infectivity when another picornavirus, BEV, was exposed to TEI. For the parvovirus, MVM the inactivation was more pronounced at pH 8 than at pH 6.

This result is in contrast to the pH-dependency reported earlier. Higher inactivation rates at low pH confirmed an inactivation mechanism based on a reaction with nucleic acids only. The results presented in this report lead to the assumption that another reaction mechanism could be involved; i.e. that a modification of viral surface proteins is also involved in the inactivation mechanism. In fact, a higher pH favours deprotonation of nucleophilic groups in proteins that would explain a faster reaction at more basic pH.

The observed inversed pH-dependency of the inactivation kinetics of the non-enveloped DNA-virus, MVM, when exposed to TEI, supports this idea. For this reason, three test proteins were exposed to TEI and changes in their pI values were monitored by isoelectric focusing. All three proteins were heavily modified by TEI. The modification with ethyleneimines led to a shift towards higher pH and a less pronounced focusing (smear) as already reported for the monomer (Budowsky, 1997, 1998). This observation can be interpreted as the introduction of additional positive charges by TEI in the target proteins. The reaction resulted in a heterogeneous population of proteins modified with different frequencies. These findings are contradictory to the reported data for human serum albumin exposed to TEI but similar to the ones observed with aziridine (Budowsky, 1998).

The hypothesis that TEI has the ability to modify proteins was further supported by the observed changes in the mass spectra for myoglobin exposed to TEI compared to the negative controls. A sequence of up to four additional peaks with a mass difference in good agreement with the TEI mass could be detected. In accordance with the results obtained in the inactivation assays with viruses, isoelectric focusing and MS analysis showed that myoglobin was less modified at pH 6 than at pH 7 or 8.

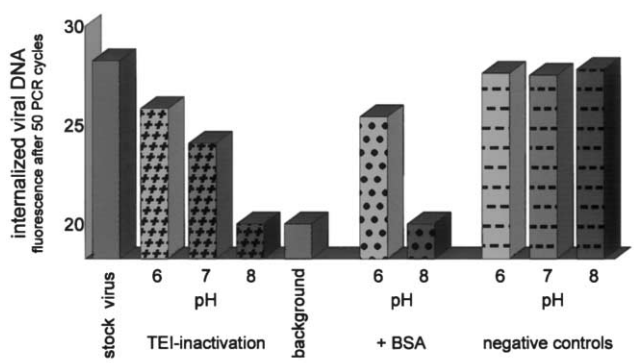

Fig. 5. Inhibition of MVM uptake by TEI treatment. Virus uptake was analysed by measuring the internalised MVM DNA by real-time PCR. Viral DNA is expressed as fluorescence intensity after 50 PCR cycles. The results from inactivation experiments performed at different pH are shown (+). Additional assays were performed in the presence of BSA (●). In control experiments, TEI was quenched prior to exposure to MVM (−). Untreated MVM stock virus was tested as positive control. Probes without virus were added to check for background fluorescence.

To obtain more information on the mechanism of virus inactivation by ethyleneimines, studies investigating the effect on virus uptake into host cells were performed. Our results show that the observed loss of MVM infectivity coincides with the inhibition of virus uptake. Furthermore, TEI-inactivated SFV showed a different protein pattern in mass spectra compared to not inactivated virus samples (data not shown). Thus, virus inactivation by TEI takes place most likely via a modification of viral surface proteins. The membrane permeability of the positively charged TEI, a prerequisite to modify nucleic acids of an enveloped virus, was not addressed in earlier reports but has to be questioned.

In conclusion, this study demonstrates that the virus inactivation procedure using TEI does not specifically attack nucleic acids without modifying viral proteins or soluble proteinaceous components in a biological system. This report shows that it is a difficult task to find virus inactivation methods that efficiently eliminate viral infectivity without interfering with the integrity of the proteinaceous (non-viral) components of the preparation.

Acknowledgements

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