

Changes Induced by Pre-Fixation in Polycation Bacteriolysis and Surface Alterations Matching the Model of Wall Picnosis in Cell Lysis

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Z. Naturforsch. **35 c**, 805–810 (1980); received July 9, 1980

Poly-L-arginine, Protamine, *Bacillus subtilis*, *Staphylococcus aureus*, Glutaraldehyde

Pretreatments of *B. subtilis* and *S. aureus* cells with lower concentrations of fixative agents, led to modifications in bacteriolytic effect exerted by polyarginine and protamine: Glutaraldehyde blocked polycation bacteriolysis while formaldehyde and osmium tetroxide (OsO_4) having no influence on polyarginine action, increased constantly the cell sensitivity to protamine in lower doses otherwise nonlytic; the sensitizing action also resulted in the extension of protamine bacteriolytic pattern including several staphylococcal strains; higher bacteriolytic doses of protamine were contrastively unable to lyse OsO_4 prefixed cells and gave an inconstant lytic value with formaldehyde treated bacteria. With higher concentrations, OsO_4 preserved intactly its sensitizing action while formaldehyde displayed a decrease in its ability to sensitize *B. subtilis* cells to the lytic effect of protamine. Scanning electron microscopy of polycation treated cells showed prelytic lesions as surface granulations, shape and size modifications and cell splits. The interpretation of the results in terms of intra- and intermolecular adducts accompanied by conformational changes in surface macromolecules is discussed. It is concluded that the results match the model of polycation bacteriolysis by wall multizonal picnosis leading to surface splits and thereby triggering cell-lysis.

Introduction

Bacteriolysis by polyarginine and protamine, predominantly ranged amongst Gram positive organisms, displayed taxonomic diversity for each polycation, the bacteriolytic pattern of protamine being less extended, and its intensity of action on the same strain significantly lower than that of polyarginine [1]. As basic mechanism of polycation bacteriolysis was assumed to be a multistep process of wall plurizonal condensation resulting in conformational dislocations of mural core polysaccharides caused by multiple salt binding interactions.

Here we describe the effects of cell pretreatments with glutaraldehyde, formaldehyde and OsO_4 on the sensitivity to polycation cell-lysis of the different strains of *B. subtilis* and *S. aureus*, the fixative agents inducing important changes either in the intensity of bacteriolysis or in its taxonomic extension. The results of tripartite subsequent interactions, fixative

agent-bacterial cells, and then polycation-fixed cells seem to provide more information about surface action of fixative agents as well as concerning the primary effect of the polycations in their ability to trigger bacteriolysis in shake conditions of incubation. Structural changes in polycation treated cells of *B. subtilis* as seen by scanning electron microscopy are also described.

Materials and Methods

Organisms, media, reagents, stock solutions and bacterial initial suspensions

Bacteria. The strains used are mentioned in the Table I.

Media and reagents. Tryptose Blood Agar Base Difco (TBAB), Penassay Broth Medium 3 Difco and minimal liquid medium [2] (abbreviated as BM) were used. For staphylococci, instead of BM that gave some irregular spontaneous instability of cocal cells in shake condition of incubation, a mixture BM/penassay broth in ratio 5/1 (BM-1) was used. The reagents were from the same commercial sources as mentioned [1].

Stock solutions. OsO_4 1% prepared in Kellenberger's buffer [4], commercial solutions of glutaralde-

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0341-0382/80/0900-0805 \$ 01.00/0



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hyde 25% and formaldehyde 37% were stored at 4 °C. Intermediary dilutions of aldehyde fixatives were performed in bidistilled water and immediately used. The preparation of protamine 2 mg/ml and polyarginine 400 µg/ml was carried out as described [1].

Bacterial initial suspensions were prepared following the protocol described previously [1], using BM for *B. subtilis* and BM-1 for staphylococci. All the initial suspensions were immediately used.

Pretreatments with fixative agents and their effects on polycation bacteriolysis

In separate series of aliquots of 1.8 ml from bacterial initial suspensions of *B. subtilis* cells, the fixative agents were added as intermediary dilutions of formaldehyde and OsO₄ stock solutions, 0.1%, 0.37%, 0.5% and 1% in portions of 0.2 ml, and glutaraldehyde 0.25%, 0.2 ml. The samples were kept at 23–25 °C for 10 min, centrifuged in a swing out rotor at 2000×*g* for 15 min, the supernatants discarded and the liquid from inner surface of the tubes soaked in filter paper packed in sticks. The pellets were resuspended in the same volume of BM and the resulted suspensions were distributed as 0.1 ml aliquots, in separate series of protamine and polyarginine dilution samples performed as described [1], the final concentrations of polycations being mentioned in the Fig. 1. The different series corresponding to presented subsequent associations of fixative agents and polycations were incubated at 37 °C for 60 min with reciprocal shaking at 120–130 strokes/min the sample tubes of 18/180 mm having a slanted angle of 30°.

The fixative pretreatment of staphylococcal strains were performed as above, but using for formaldehyde and OsO₄ only the intermediary dilutions of 0.37% and 1% respectively, BM-1, incubation with fixative at 37 °C, and polycation concentrations mentioned in the Table I.

Control samples for each strain were: Fixative untreated bacterial suspension free of polycation; fixative treated polycation free bacteria; fixative untreated bacteria mixed with polycation (Table I). All of them were prepared, incubated and examined as with assay samples were done.

The estimation of bacteriolysis was carried out by OD₃₄₀ measurements and phase contrast microscopic examinations [1]. The use of OD₃₄₀ instead of

OD₄₉₀–OD₅₅₀ measurements (e.g., [5]) was justified by higher sensibility as well as by the fact that BM, BM-1 and polycation solutions referred to distilled water did not give any absorbance value at λ of 340 nm.

Conditions for scanning electron microscopy of B. subtilis cells passed by OsO₄-protamine cycle or treated with polyarginine alone

Cultures of strain WT of 16 h at 37 °C on slant TBAB were suspended, washed once by centrifugation and resuspension in Kellenberger's buffer [4], and quantified at OD₃₄₀ = 2.1. An aliquot of 5.4 ml was then completed with OsO₄ 1% 0.6 ml, kept for 10 min at 23–25 °C and centrifuged at 2000×*g* for 15 min. The pellet was resuspended in 122 ml of buffer [4] and poured as 59 ml aliquots in two Erlenmeyer flasks of 500 ml. These were completed, one with protamine stock solution 1.2 ml, and the other with bidistilled water 1.2 ml. In preparing polyarginine sample, buffer [4] 52 ml, bacterial suspension (OD₃₄₀ = 2.1), 2.5 ml and polyarginine stock solution 6 ml were mixed in an Erlenmeyer flask of 500 ml. The samples were then incubated at 37 °C for 20 min with reciprocal shaking at 120 strokes/min, centrifuged at 3000×*g* and 2 °C for 60 min, the resulted pellets being used in the preparation of specimens for scanning electron microscopy (SEM). The specimens were processed following the protocol of glutaraldehyde fixation, OsO₄ postfixation subsequent ethanol dehydration and chrom coating as described [6]. SEM examinations were undertaken using JEM JEOL type 100 C, operating at 20 kV.

Results

As seen by OD₃₄₀ measurements and phase contrast examinations, the pretreatments of *B. subtilis* and staphylococcal cells with glutaraldehyde 0.025% resulted in the loss of their sensitivity to polyarginine bacteriolysis the lytic effect of 200 µg/ml protamine on *B. subtilis* cells being also blocked. In contrast to glutaraldehyde action, the pre-fixation of *B. subtilis* cells either with formaldehyde 0.037% or with OsO₄ 0.1% did not change the bacteriolytic effect of polyarginine previously described [1] while protamine bacteriolysis underwent significant alterations. Plotting the OD₃₄₀ values of prefixed bacterial samples against protamine concentrations,

Table I. Pattern of polycation bacteriolysis of a set of 21 strains of *S. aureus* expressed as average values of OD₃₄₀ of different groups of strains, calculated from different mean values of three different assay samples for each strain. All staphylococcal strains having usual designations were provided as mentioned [1]. + = Strains of *B. subtilis* taken as control of polycation lytic abilities; their origin was previously described [1, 3].

Polyarginine 40 µg/ml	Protamine 200 µg/ml	Formalin Protamine 80 µg/ml	OsO ₄ Protamine 40–80 µg/ml	Strains
0.03	0.22	0.08	0.05	S. 42 B/42 C; S. 6; S. Cowan
0.05	0.24	0.21	0.04	S. Oxford; S. Wood; S. 47; S. 81; S. 3 C; S. 77; S. 83 A
0.05	0.27	0.20	0.16	S. 52; S. 54; S. 71; S. 53; S. 89; S. 80; S. 75; S. 88
0.06	0.21	0.10	0.20	S. 7; S. 52 A/79; S. 29
0.03	0.06	0.05	0.04	+WT; +861

the formaldehyde pretreated and OsO₄ pretreated bacteria had maximal lytic sensitivity to protamine 80 µg/ml and 40 µg/ml, respectively, the fixative untreated bacteria being not lysed by the same concentrations of polycation (Fig. 1). With higher concentrations of protamine the lysis of fixative treated bacteria decreased progressively while the same polycation doses were efficiently lytic for unfixed cells. Confidence limits of the last sample of formaldehyde series, illustrates the lonely irregular distribution of OD values amongst 10 different assays, this variance being not displayed by the other samples in the series. Although the pretreatments with different concentrations of OsO₄ between 0.01% and 0.1% resulted in invariable enhancement of bacteriolysis by protamine 40 µg/ml, the same pretreatments with formaldehyde (Fig. 1) had different effects, in higher concentrations being less efficiently in increasing the lytic ability of protamine 80 µg/ml.

The strains of *S. aureus* were assayed in screening for a possible strain diversity. Starting with OD₃₄₀ = 0.28–0.30 for all strains, only the decrease of OD at least to 0.10 was considered to be significant in stating on clear-cut expression of the bacteriolysis for screening. As Table I illustrates, albeit all the staphylococcal strains invariably were polyarginine lytic and protamine nonlytic, the pretreatments with formaldehyde 0.037% or OsO₄ 0.1% resulted in a diversity of four types of sensitizing to protamine bacteriolysis as follows: (i) Formaldehyde and OsO₄ sensitizing – 3 strains; (ii) formaldehyde unsensitizing and OsO₄ sensitizing – 7 strains; (iii) formaldehyde and OsO₄ unsensitizing – 8 strains; (iv) for-

maldehyde sensitizing and OsO₄ unsensitizing – 3 strains.

Since orientative preliminary assay showed that polycation bacteriolysis was produced with the same efficiency in Kellenberger's buffer, this was used in preparing bacterial pre-lysing specimens for SEM examination. SEM micrographs of polyarginine treated bacilli showed pre-lysing alterations as pro-

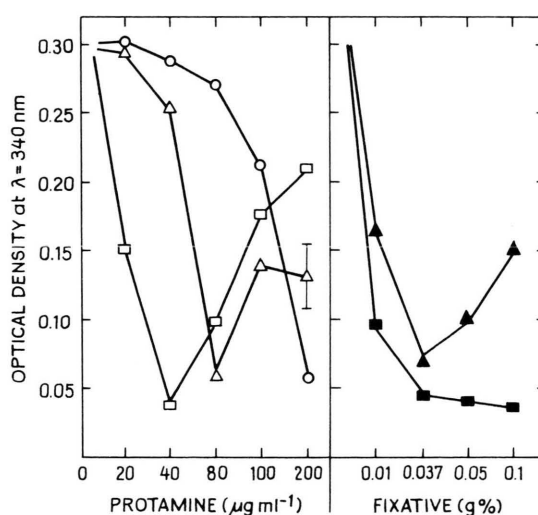


Fig. 1. Protamine bacteriolysis illustrated by plotting the changes in OD₃₄₀ values of bacterial samples with fixative untreated cells (○), formaldehyde 0.037% pretreated cells (△) and OsO₄ 0.1% prefixed cells (□). Sample series having bacteria prefixed with different concentrations of formaldehyde (▲) and OsO₄ (■), and subsequently exposed to protamine 80 µg/ml and 40 µg/ml, respectively. *B. subtilis* strain WT was used. The polyarginine series of samples with concentrations of 10, 20, 40, 60, 80 µg/ml and prefixed bacteria had the same profile of bacteriolysis as described [1].

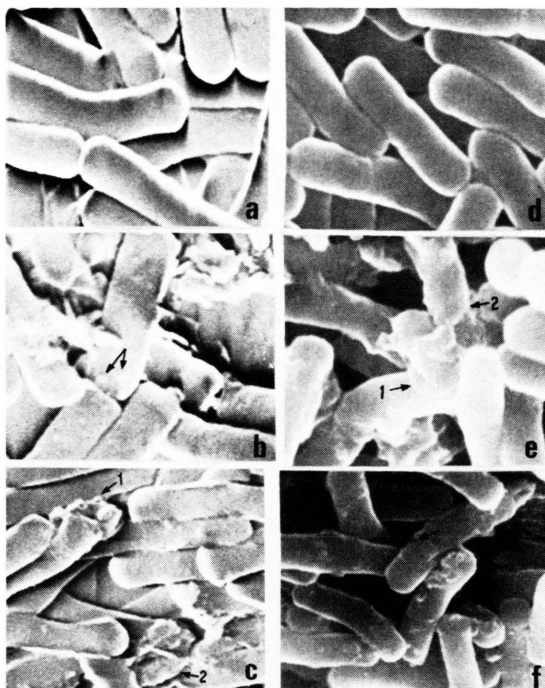


Fig. 2. Cell-surface alterations caused by polycations in pre-lysed steps. The SEM micrographs, in conditions of high contrast (a, b, c) and normal contrast (d, e, f) were performed as described [7]. Control polycation free specimens (a, d) (magnification 38 000 \times). Polyarginine treated bacteria: b = cells with surface granules (\rightarrow) and cell fragments condensed in clusters (top) (39 000 \times); c = bursting split (1) and irregular cell contours (2) (29 000 \times). OsO_4 prefixed-protamine treated cells: e = contorsioned cell (1) and serrated contours (2) (29 000 \times); f = several surface granules of different size, and splits (\rightarrow) (17 000 \times). *B. subtilis* strain WT was used.

truded surface granules, irregular bacterial contours, and splits of different size and form. The surface of polycation treated cells was rough and contrastive with smooth aspect of surface of the control cells. The rough profile might correspond to a high diversity of the size of surface granulation culminating with clear-cut protruded granules indicated in the Fig. 2. Besides the cells not yet lysed there were cellular clustered fragments possibly resulted from bacteriolysis already produced. As control micrographs illustrate (Fig. 2), OsO_4 prefixation alone did not induce significant alterations in SEM morphology, but followed by protamine action, the fixative pretreated cells showed important changes in their surface structure similar with those induced by polyarginine treatment alone.

Discussion

Although glutaraldehyde and OsO_4 are considered as fixative agents with similar effects causing fine precipitations of proteins without appreciable structural distortions [8], their actions on polycation bacteriolysis are quite different. The ability of glutaraldehyde prefixation to stop both polyarginine and protamine cell lysis may be provided by its property to polymerize spontaneously even in commercial solutions [9] forming multifunctional polymers and thereby having the capacity to establish long cross bridges between amino groups belonging to different constituents of the bacterial envelope. In this multilayered structure, the glutaraldehyde having several degrees of polymerization could form at least two types of adducts: Intra-molecular proteinic and intraglycanic adducts established particularly by simple and oligomeric forms; intermolecular long distance cross links between different surface macromolecules as membrane proteins and wall polysaccharides (peptidoglycans, amino-group bearing teichoic acids). Intermolecular adducts increase the envelope stability, form a network of glutaraldehyde polymeric cross bridges able to block the continuous surface conformational changes proposed as mechanism of the environmental interactions [10], and thereby impeding the polycation molecules to interact with their surface sites.

Albeit the OsO_4 affinity for lipids [11] might account for bacterial surface modifications, as heavy metal compound, OsO_4 could interfere with tertiary structure of proteins [12] inducing stabilized conformational changes in surface proteins and resulting in the enhancement of polycation bacteriolysis. Thus OsO_4 , forming no intermolecular cross-adducts of glutaraldehyde type, it interacts with proteins, gives fine precipitations and favours the polycation-mural polysaccharide interactions.

The heterogeneity of formaldehyde effects on polycation bacteriolysis may be explained by both reversible and irreversible steps of interactions with amino bearing constituents [13]. With low concentrations (0.037%) formaldehyde, acting predominantly as monofunctional agent, forms activated reversible groups of the type $\text{R}-\text{NH}-\text{CH}_2\text{OH}$ and may induce OsO_4 like conformational changes which result in the increase of cell sensitivity to polycation action. With higher concentration (0.1%), it may

have increased probability to act as bifunctional agent, may form irreversible cross adducts of the type $R-NH-CH_2NH-R'$ (R and R' being different amino-group bearing macromolecules), and thereby becoming less sensitizing for protamine bacteriolysis (Fig. 1). Thus the reversible first step would favourize the intimate penetration of polycation molecules into the cell envelope structure, and their lytic interactions. This first step of formalin action, inducing an initial increase of surface permeability, may account for the higher and faster penetrability of the formaldehyde [12], the primary reversible interactions favourizing its own molecules to penetrate deeper in the cells and tissue blocks.

The results of SEM examinations (Fig. 2) match clearly enough the model of polycation bacteriolysis by wall multizonal condensations [1] because the surface alterations may be the results of condensing forces developed by multiple salt binding associations illustrated as rough surfaces, multisite granulations and surface splits. The intensity and the extent of wall-polycation condensation in its multihit running, appear to be very high since there were bacillary cells with deep changes in the entire bacterial shape consisting in contorsioned bacilli, extended splits and condensed fragments aggregated in tight clusters.

The effects of fixative pretreatments also match the model of mural multisite condensation as follows: (i) The long intermolecular adducts formed by oligo- and polymerized states of glutaraldehyde, stabilizing the surface constituents at a high degree, impede the polycation-wall interactions and wall splitting alteration; (ii) the conformational changes induced by OsO_4 open the pathway of more intimate interactions between polycation molecules and wall core polysaccharides, a similar effect exerting also formaldehyde in the monofunctional step of its action; (iii) the second bifunctional step occurred significantly with higher concentrations resulted in

the decrease of formaldehyde ability to open the way for polycation condensation. Since OsO_4 acts as an irreversible single step agent, its lower dose effect was preserved with higher concentration too (Fig. 1); (iv) inability of OsO_4 and formaldehyde prefixations to change polyarginine bacteriolysis as a result of higher capacity of this polycation to produce more extended wall condensations [1] which surpass OsO_4 and formaldehyde alterations.

The reverse effects of OsO_4 and formaldehyde pretreatments on lytic action of higher concentrations of protamine appear difficult to be explained. However, these higher protamine concentrations could favourize a state of site competitiveness amongst interacting polycation molecules resulting in the decrease of polycation ability to lyse the OsO_4 and formaldehyde prefixed cells otherwise lysed by lower protamine doses. The irregular effect exerted by formaldehyde prefixation on bacteriolysis with 200 $\mu g/ml$ protamine, could be provided by more complex distribution of the two steps type of interactions:

The intra-species diversity of staphylococcal bacteriolysis by protamine displayed merely in OsO_4 and formaldehyde prefixed conditions, could be explained by the surface structure polymorphism classically demonstrated by heterogeneous pattern of phage lysotyping, antigenic serotyping (*e.g.*, [14]), and by strain structural diversity of peptidoglycan molecules [15]. The last heterogeneity together with prefixed cell-protamine bacteriolysis displaying a pattern of four types (Table I), could be considered as an indirect evidence suggesting peptidoglycans complexed with teichoic acids [16], as sites of polycation bacteriolysis interactions.

Acknowledgements

We thank Dr. Al. Petrovici and Dr. G. Gancevici, Cantacuzino Institute Bucurest, for their helpful suggestions and preliminary discussion.

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