Polycation-Bacterium Interactions and Wall Subunits as Endocytosis Factors. Topoisomeraselike Action of Basic Polypeptides Suggesting a 7th Class of Enzymes: The Stereases

Stefan Antohi

Institut de Virusologie St. S. Nicolau, Mihai Bravu 285, 79650 Bucarest, Romania

Lysine rich histone and polylysine are bacteriolytic for *Bacillus subtilis* and *Escherichia coli* cells, whereas histones H2A, H2B and H3, assayed separately are not. Polyarginine bacteriolysis of *E. coli* with polycation 10 µg/ml decreased with higher concentrations, the killed nonlysed cells exhibiting wall alterations. All bacteriolytic polycations were lytically effective under reciprocal shaking, but not under magnetic stirring conditions, demonstrating the requirement for cell lysis of mechanical fields developed in oscillatory movements. Scanning electron microscopy of histone H1 treated *B. subtilis* cells showed the formation of globular bodies (polionosomes) in the cell surface. The interference of Ca²⁺ and 0 °C exposure with the polycation action observed especially in *E. coli*, the polionosomes, and the dynamics in polycationic cell lysis, considered together, suggest the existence of wall supramolecular subunits (murameres), defined by their ability to form polionosomes. The murameres undergoing reversible conformational transitions induced by ectobiological signals, *e.g.*, Ca²⁺, would make up an uptake system. The involvement of this system in environmental interactions and DNA uptaking, as well as the general stereo-enzyme like nature of the conformational changes caused by polycations are discussed.

Introduction

The multizonal wall condensation caused by polyarginine and protamine, similar in its mechnism with chromatin DNA pycnosis [1, 2], leads to different working hypotheses. For instance, there might be a heterogeneity in different histone polycations (H1 and inner-nucleosomal histones) when interacting with the bacterial cell surface. Cell surface – polycation interactions resulting in bacteriolysis are conditioned by reciprocal shaking [1]. This oscillatory motion of the samples may be, an energy source of cell lysis while a rotatory motion (*e.g.* magnetic stirring) may fail to promote cell lysis. The cell wall of almost all bacteria maintains cellular integrity in spite of inner osmotic forces (the tensile strength of the wall preventing disruption by osmotic shock [3, 4]). Hence the investigation of surface modifications occurring during the prelytic stages of polycation bacteriolysis might suggest a subunit structure of the wall core, with possible fast transient changes of the mural ensemble engaged in fulfilling its own functions. Accordingly, the wall multizonal pycnotic modifications induced by histone H1 and revealed by scanning electron microscopy in the form of defined globular structures would suggest the existence of functional wall subunits having the ability of undergoing globular changes amplified and stabilized by the polycation. Under polycation-free ectobiological conditions the globularization of these wall subunits would be of rapid reversal and therefore difficult to be revealed structurally. Here are described some experimental findings and theoretical considerations that support the above assumptions leading to a model of cell surface modifications occurring in the ectobiological interactions of the bacterial cell, including nutrient metabolic exchanges and DNA uptake in genetic transformation, transfection and DNA cloning. Data regarding polylisine action, Ca²⁺, and low temperature treatment in correlation with polycation effects are also reported.

Materials and Methods

1. Bacteria

B. subtilis strain 861 [1] and *E. coli* strain PRC 399 [5] were used. For comparative assays of Gram negative bacteria, routinely isolated wild types of *E. coli* – 34 strains, *Pseudomonas aeruginosa* – 10 strains, *Salmonella typhi murium* – 5 strains and *S. enteritidis* – 5 strains (kindly provided by Dr. Ina Butnaru, Antiepidemic Center of Bucarest) were employed.

2. Media, reagents, stock solutions and initial bacterial suspensions

The strain 861 was routinely grown as described [1]. The other strains were grown on tryptose nutrient agar (Merck). From Sigma were: protamine sulfate; poly-L-lysine hydrobromide, MW 13000; poly-L-arginine hydrochloride, MW 60000; lysine rich histone − III S (H1); slightly lysine rich histones − VI S(H2A) and − VII S(H2B); arginine rich histone VIII S(H3). All other reagents were from Merck.

Stock solutions. $CaCl_2$ 2 M; acetate-saline (natrium acetate 40 mM, NaCl 150 mM, pH 5.6); glutaraldehyde 25% for electron microscopy; OsO_4 1% prepared as described [2]. All were stored at 4 °C. Separate polycation solutions 2 mg/ml in bidistilled water were kept in portions of 2 ml at −10 °C and used within a 12-day interval. The haloidacid derivatives of the synthetic polycations were easily dissolved even in this higher concentration.

Initial bacterial suspensions were prepared as described [1] by substituting the respective medium with acetate − saline and used instantly.

3. Conditions of polycation cell lysis using B. subtilis, E. coli PRC 399 and other Gram negative bacteria

Separate sample series corresponding to each polycation and to each strain were prepared as described [1] except for some changes: the respective medium was replaced by acetate-saline; for lower concentrations (Fig. 1), stock solutions of polycations were prediluted to 400 µg/ml, and for higher concentrations (up to 100 µg/ml) their sample ranges were extended with samples corresponding to the protamine series [1]; polyarginine hydrochloride not giving precipitates in acetate-saline, the shake preincubation was precluded; incubation time was of 30 min. Samples of the wild type strains were likewise prepared in the polyarginine assay, but only with concentrations of 10 µg/ml and 100 µg/ml.

4. Assays of the polycation lytic action under conditions of magnetic stirring (circular motion) versus reciprocal shaking (oscillatory motion)

For each polycation and each strain, separate sample ranges were prepared by pipetting 18-ml aliquots of acetate-saline in two Erlenmeyer flasks of 200 ml (bottom diameter $\simeq$ 80 mm) and in two 80-mm Petri dishes, and by distributing 1 ml initial bacterial suspension per aliquot. The Petri dishes were of Jena glass type with a smooth levelled inner surface of the bottom. From each pair of samples, one sample was completed with polycation stock solution to the final concentration having a maximal bacteriolytic effect (Fig. 1) and to a final volume of 20 ml; the other, completed with distilled water, was used as control sample. The Erlenmeyer flask samples were incubated at 37 °C with reciprocal shaking at 120 strokes/min (harmonic motion) for 30 min. The Petri dish samples were incubated under magnetic stirring (circular motion) using glass-covered rods of 3/25 mm at about 200 rotations/min for 60 min. Only *E. coli* strain PRC 399 and *B. subtilis* were used.

5. Conditions for assaying the influence of Ca^{2+} and 0 °C on the actions of polyarginine and polylysine

Series of 3 samples for both *E. coli* PRC 399 and *B. subtilis* were prepared by distributing acetate-saline 1.8 ml, initial cell suspension 0.1 ml and $CaCl_2$ 2 M 0.1 ml in tubes of 18/180 mm, and keeping them continuously in the ice bath. After 30 min, polyarginine 10 µg/ml and polylysine 20 µg/ml were added to two different *E. coli* samples while two *B. subtilis* samples received 40 µg/ml each polycation, respectively. The sample tubes were fastened in the ice bath at an angle of 30°, the ice bath container was fixed on a reciprocal shaker and then incubated under shaking at 120 strokes/min for 30 min. The temperature of the samples was then quickly shifted to 37 °C in a warm water stream and incubation continued for 15 min at 37 °C with shaking as described [1]. Parallel Ca^{2+} free series of samples with identical temperature shift were prepared. Control series of samples for both bacteria and for both polycations, with and without Ca^{2+}, were similarly prepared, with the exception that they were kept 45 min at room temperature and then incubated for 15 min under shaking as described [1]. Other Ca^{2+} free control series of polycation-bacterial mixtures were incubated for 15 min under shaking immediately after addition of the polycations.

6. Quantitation of polycationic bactericidal and bacteriolytic effects

Aliquots of 0.1 ml from different samples were adequately diluted and plated quantitatively on tryptose agar with a view to score the survivors as

CFU (colony forming units) per ml. Bacteriolysis was estimated by OD_{340} measurements [1] and phase contrast microscopy.

7. Challenge of polyarginine superdosage – killed nonlysed cells of E. coli by the osmotic shock under static and shake incubation

Six samples of the strain PRC 399 were prepared as described in Section 3, containing polycation 200 µg/ml. After shake incubation as described [1], the six samples were collected in a centrifuge tube, pelleted, washed twice with acetate-saline, resuspended in distilled water 12 ml, redivided into six samples of 2 ml and kept statically at 23 °C. At intervals of 30 min, 60 min and 18 h, the OD_{340} values were estimated before and after shake reincubation for 30 min as described [1].

8. Scanning electron microscopy (SEM) of B. subtilis cells treated with lysine rich histone

Samples pretreated with OsO_4 0.1% were completed with lysine rich histone 100 µg/ml and incubated with shaking, according to the routine previously described [2]. The examination of SEM specimens was undertaken by using a TEM/SEM Philips Type EM 400 at 20 kV (Fig. 2).

Results and Discussion

1. Diversity in the interaction of different polycations with the cell surface, inferred from their heterogeneous bacteriolytic efficiencies and lytic free bactericidal effects

As estimated by OD_{340} and phase contrast microscopy examinations, lysine rich histone (H1) caused bacteriolysis of B. subtilis cells, the decrease in turbidities with increase in polycation concentration being very similar to that described for protamine [1]. Although histone H1 was less efficient in E. coli cells (Fig. 1), the decrease in turbidities may be considered as significant because the OD values were similar along six different assays. Protamine exerted its lytic effect on E. coli at lower concentrations than those efficient for cell lysis in B. subtilis [1]. In contrast to histone H1, the inner histones H2A, H2B and H3 taken separately were unable to lyse either B. subtilis and/or E. coli cells. This contrast in bacteriolytic efficiency between H1 and inner histones may be explained by the analogy of the
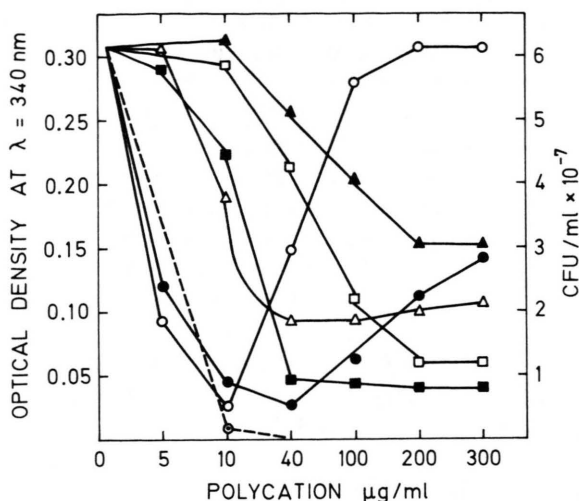

Fig. 1. Diversity in polycation bacteriolysis plotted as OD_{340} values against polycation concentrations. B. subtilis strain 861 lysis: polylysine (■); histone H1 (□). E. coli strain PRC 399 lysis: protamine (△); histone H1 (▲); polyarginine (○); polylysine (●). E. coli survivors (– – ○ –) in the polyarginine series. Separate series of histones H2A, H2B and H3 mixtures with E. coli and B. subtilis cells, respectively, gave no changes in initial $OD_{340} = 0.30$.

mechanism of polycationic cell-wall condensation with that of chromatin DNA pycnosis, suggested previously [1]. Since inner histones interact only together as $(H2A\ H2B\ H3\ H4)_2$ octamers in DNA nucleosomal compaction [6, 7] the separate fractions H2A, H2B and H3 were not able to condense zonally the cell wall, being nonlytic. Histone H1, which participates alone in chromatin super-packing [8, 9], caused wall polycationic condensation being lytic. Thus, in addition to the previous observations [1, 2] the results reported here appear to fit once more into the model of multizonal wall pycnosis in polycation bacteriolysis and its mechanism analogy with chromatin DNA pycnosis.

Polylysine induced B. subtilis cellular lysis demonstrated a drastic decrease of OD_{340} to 0.04 at concentrations of 40 – 300 µg/ml (Fig. 1), while in E. coli samples the polylysinic cell lysis displayed a superdosage increase of OD values corresponding to a decrease in bacteriolysis, also observed in phase contrast microscopy examinations. This superdosage "rescue" to polycation bacteriolysis was more illustratively expressed in the polyarginine series of E. coli: as Fig. 1 shows, polyarginine gave a maximal value of lysis at a concentration of 10 µg/ml, the

concentrations up to $20 \mu g/ml$ corresponded to a progressive decrease of bacteriolysis, the highest concentrations being nonlytic. As the hyper-dosage "rescue" was less displayed by polyarginine in the *B. subtilis* series (not illustrated), it was tempting to think that the surface structures as outer membrane, periplasmic space components and the lipopolysaccharide richness missing in *B. subtilis* but present in *E. coli* envelope [10, 11], would rapidly interact with hyperconcentrated polyarginine molecules. This would result in reorganized polycationic surface layers which hinder cell lysis, possibly being disfunctional. Scoring the *E. coli* survivors in the polyarginine series (Fig. 1) we found a survival of about 10^4 CFU/ml at the concentration with maximal lytic value, while the higher, less bacteriolytic or nonbacteriolytic concentrations were strongly bactericidal, no viable cells being observed, which suggests envelope disturbances incompatible with cell survival. Routine examination of the Gram stained slides of *E. coli* cells prepared from the pellets of samples containing polyarginine $100-300 \mu g/ml$ washed once with acetate-saline showed a significant decrease in the staining with basic fuchsin, and the appearance of a slight, but evident affinity for acid stains, such as acid fuchsin and azocarmin B. As compared with the staining affinities of control samples there was a suggestive shift, from basic to acid staining affinity, also pleading for a cell surface modification. The wall reorganization was further suggested by the behaviour of polyarginine superdosage killed cells subjected to osmotic shock by resuspension in distilled water. Killed bacteria, as well as the control samples not treated with polyarginine, resisted in distilled water, even if maintained statically at $23 °C$ for 18 h, having an $OD_{340} = 0.24 - 0.30$. When incubated at $37 °C$ with reciprocal shaking as shown above, polyarginine killed bacteria were drastically lysed, the OD value dropping to $0.02 - 0.06$ while the control sample remained unchanged. Shake-conditioned cell lysis of superdosage polyarginine killed bacteria relieved of polycationic excess by washing, also occurred at different postwashing intervals. This indicates that the envelope core has been highly altered but it reorganized since it resisted the statical osmotic shock like that of normal control bacteria. Such postwashing shake induced bacteriolysis was also observed when polyarginine sample pellets were washed and resuspended only in acetate-saline. The

reorganized wall had fixed a small quantity of the excessively dosed polycation, since the polyarginine supernatant 1/20 diluted, challenged with the initial cell suspension at $OD_{340} = 0.30$ and shake incubated resulted in an OD decrease to 0.02. This shows the insignificant polycation amount fixed by the first lytic free killed cells removed by centrifugation.

The lytic effect of lower polyarginine concentrations on Gram negative *E. coli* cells is apparently in contrast to the previously reported data [1]. In that work, however, Gram negative bacteria were tested against an invariable polyarginine concentration of $40 \mu g/ml$, which was practically nonlytic on that occasion too. Moreover *E. coli* was not assayed previously [1]. Rechallenging polyarginine $10 \mu g/ml$ against the 54 strains routinely isolated in laborator diagnosis, the decrease of the initial $OD_{340} = 0.30$ below 0.15 was present in: 22 strains of *E. coli;* 5 strains of *Pseudomonas;* 3 strains of *S. typhi murium.* The other strains were nonlytic. These results would suggest some reconsiderations concerning the diversity of polycation bacteriolysis in Gram negative bacteria, indicating *Escherichia* as a higher sensitive taxonomic group. All the lytic strains displayed polyarginine superdosage lysis inhibition, but showed a quantitative diversity in OD values and gave no survival values.

The evident differences in the superdosage inhibiting effect between polyarginine, polylysine and protamine (Fig. 1) may be due to the greater molecular length of the polyarginine chain and to the close reiterate guanidinyl groups of arginine residues. The larger register of noncovalent interactions of the guanidinyl group [12] as compared with that of the amino group would account for the difference in binding, leading to a possible wall molecular rearrangement, able to ensure the maintenance of cell integrity in spite of the killing alterations.

The ensemble of the Fig. 1 also shows a significant discrepancy between the effects exerted by natural and synthetic polycations, the latter being generally more active; this illustrates once more the differences between the interaction abilities of chemically synthesized and those of the natural polypeptides as previously defined [13]. The inner histones have been "educated" by evolution to form characteristic compact structures and thereafter to interact preferentially with double stranded DNA only when grouped as octamers; therefore they failed to pro-
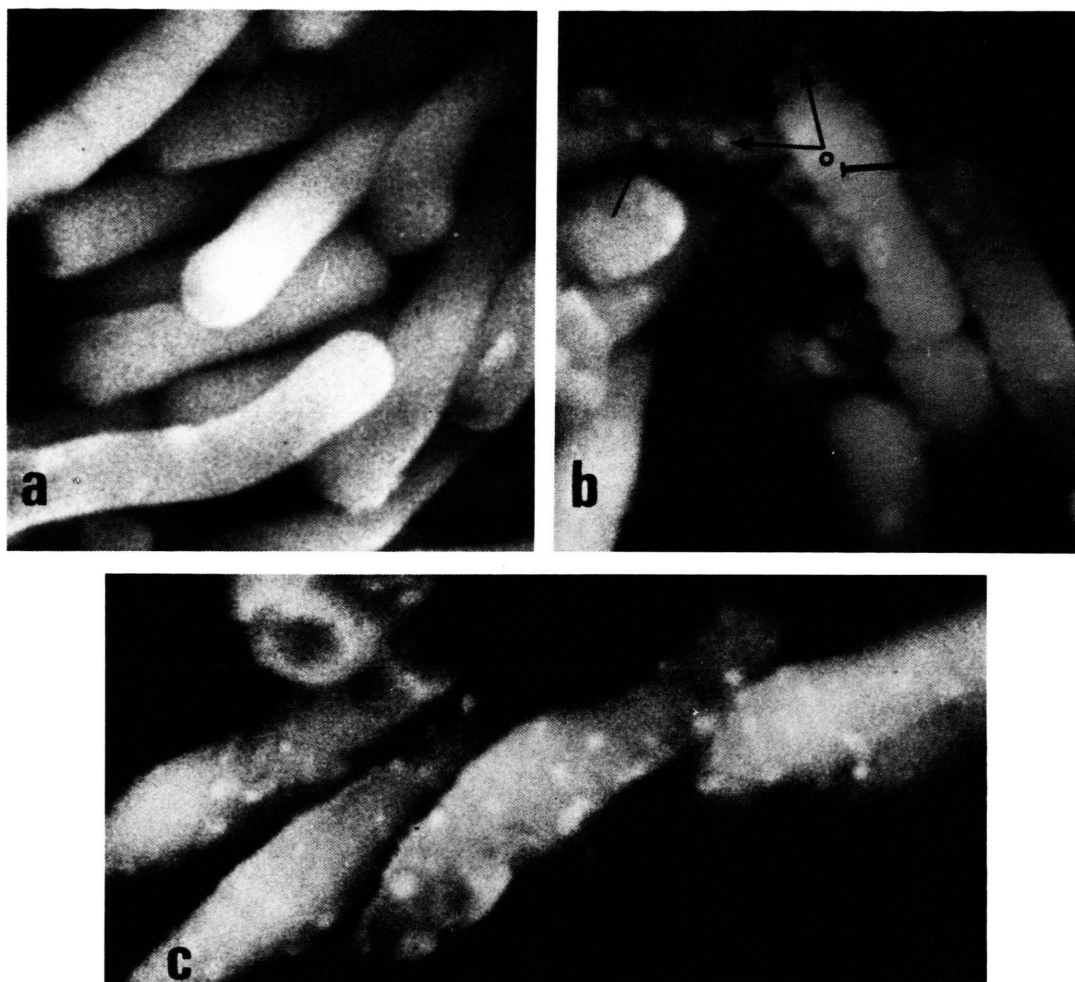
Fig. 2. Globular bodies (polionosomes) in *B. subtilis* cell surface caused by lysine rich histone. Control histone free bacteria (a). Histone treated bacteria (b): polionosomes (○→); interpolionosomal pits (→); the barr = 1 μm (magnification 12 500 ×). Histone treated bacteria (c): relative homogeneity of polionosome distribution in cell surface (25 000 ×). The chrom coating performed by V. Ciocnitu [2], was about 100 Å. SEM examinations were done in the Institute of Cell Biology and Pathology, Bucarest (by the kindness of Dr. Maya Simionescu).

mote bacteriolysis when taken separately. Since histone H1 and protamine have "learned" along the evolution scale to interact as monomers with polyanionic DNA, each of them is able to condense zonally the cell wall and to induce cell lysis, as predicted by the model of multizonal wall pycnosis [1], However, their binding abilities in wall condensations are lower than those of the synthetic polycations, which bind nonspecifically many proteins and natural structures [14]. It seems that the evolutionary organization of histone and protamine structures resulted in an absent or lower binding ability [13],

these considerations being illustrated by the diversity of the natural polycations in their abilities to induce bacteriolysis.

2. Globular surface bodies (polionosomes) as seen by SEM, and shake efficiency in polycation bacteriolysis versus stirring unefficiency suggesting the existence of conformational wall subunits – murameres

Taking into account the previous SEM examinations [2], the natural origin of histone H1 and other experimental conditions, only prelytic mixtures of histone H1 and *B. subtillis* cells were used in prepar-

ing SEM specimens. The primary prelytic alterations observed by SEM (Fig. 2) consisted in globular surface bodies very similar to those induced by polyarginine and protamine [2]. Since the resulting globular structures might be formed by interactions involving polyions, *i.e.*, wall polyanionic polysaccharides and exogenous polycation molecules, they were operationally designated as *polionosomes* (poly-ionosomes). As illustrated in Fig. 2, polionosomes are spheroid protruded bodies with a diameter of 150 – 250 nm, delimitated by pit interspaces. Their morphopoiesis assumes processes of polycation condensation binding accompanied by high macromolecular conformational changes at large distances, compulsorily causing breakages of chemical bonds. Since polycation condensation binding apparently is of the noncovalent type (salt and possibly hydrogen bonds [12]), it seems less probable from the energetical viewpoint that the pycnotic changes involved in polionosome formation, may result in the breakage of large ensembles of covalent bonds between wall components. Apparently it is more probable that during the zonal pycnotic conformational distorsions, polycationic condensation would break up low energy bonds, disassembling zonal wall parts preexisting as wall noncovalently linked structural subunits. These usual plate subunits would naturally have complementary wall-traversing interfaces, as extended sites where they interact with each other establishing large ensembles of low energy bonds. Being such large groups the latter would be energetically able to ensure the resistance of the bacterial envelope to different factors, *e.g.*, endocellular osmotic forces. The polycations, unbalancing the distribution of interface noncovalent bonds under shake incubation condition, condense polionosomes with corresponding tandem pits of low resistance. When the pits become large enough, cell lysis appears by the constant action of the endogenous osmotic pressure.

The essential requirement of reciprocal shaking in polycation cell lysis fits better the assumed existence of large wall subunits interacting pycnotically with polycation molecules in polionosome formation, than that of a lysozyme-like covalent breaking action. The "to and fro" shaking motion can be assimilated to that of the harmonic oscillator. Because such a sinusoid motion is highly non-uniform, considerable frictions are to be expected between the neighbouring strata of a fluid contained in a shaking sample even at low frequency (120 strokes/min). The fluid mechanical frictions between the cell surface and the surrounding liquid strata occurred in shake incubated polycation-bacterial mixtures would provide an important part of the energy required for the progressive protruding condensation of the nascent polionosomes. This view seems to be supported by the fact that no cell lysis was observed in the samples incubated with rotatory uniform motion, where all the mixed components moved solidarily and consequently no significant friction could be expected. The samples in Petri dishes, after 60 min of incubation had $OD_{340} = 0.22 - 0.24$ in polyarginine samples and $OD_{340} = 0.25 - 0.27$ in polylysine samples of both *E. coli* and *B. subtilis*, the polycations being practically nonlytic. The similar samples in the Erlenmeyer flasks shake incubated for 30 min had an $OD_{340} = 0.03 - 0.06$, both polycations being lytic for both bacteria. This discrepancy between shaking and stirring effects matches better the model of polycation interactions with large subunits in producing cell lysis, than that of the narrow site by site action of lysozyme inducing bacteriolysis. This known enzyme, acting on very close and uniformly distributed sites had the same lysis effect under either shake or stirring incubation.

The dynamics of polycation bacteriolysis described here, the characteristics of cell lysis pointed out previously [1], and the relative homogenous distribution of polionosomes on the cell surface in prelytic stages (Fig. 2), suggest that the murein framework of the cell wall would not be a covalently linked wall core regarded as a single large sacklike molecule [15, 16] but it would be more likely formed by subunits designated here as *murameres*. The muramere may be defined as the wall core conformational subunit able to initiate the formation of one polionosome by polycation condensation. It is linked to similar adjacent murameres by ensembles of low energy bonds distributed between complementary surfaces that possibly cross sinuously the wall thickness. It is tempting to regard the murameres as being similar to the membrane subunits described previously [17] on the basis of the ultracentrifugal homogeneity of the fragments obtained by sonication of pure membrane specimens (the term "membrane" used there also included the cell wall [17]).

In spite of the important differences in envelope structure [10, 11, 16] and in the absence of poliono-

some SEM examinations in *E. coli*, several similarities in polycation bacteriolysis (Fig. 1) and the common dynamics of friction forces in cell lysis exhibited by both *E. coli* and *B. subtilis* cells, lead to the concept that the murein core of Gram negative bacteria would be also organized as noncovalently-linked conformational subunits — murameres.

It may be concluded that many of the aspects described previously [1, 2] and the facts mentioned here make it highly unlikely that polycation action should be accounted for by the model of the enzyme-like activity breaking covalent bonds. Accordingly, the wall core might not be covalently linked as a single large sacklike molecule [16, 18], but as a sacklike multimurameric super-macromolecule in which murameres, as covalently constructed subunits, are linked to each other by large groups of low energy bonds. Nevertheless, if compared with some kind of enzyme action, the polycation activity in bacteriolysis would appear to be analogous to the recently described DNA topoisomerase activities relaxing noncovalent binding and favouring DNA unwinding process (*e.g.*, [19]). Similarly polycation action might relax intermurameric noncovalent binding, thereby initiating polionosomal condensation and formation of large wall pits, finally resulting in cell lysis.

3. Factors suggested to represent another class of enzymes – that change steric conformations, break and/or restore noncovalent bonds, without changing the covalently stabilized intramolecular topography

The high molecular conformational changes in polionosomal pycnosis and their comparison with those caused by the action of DNA topoisomerase suggest a general hypothesis regarding some basic common traits of the steric conformational changing factors. Although some DNA topoisomerases, such as *E. coli* DNA gyrase [20], catalyse steric conformational changes of the DNA duplex energetically dependent on ATP breakage, they are basically different from isomerases [21] and other international classes of enzymes (*e.g.*, [16]). DNA topoisomerases change the steric configurations possibly breaking up and/or restoring noncovalent bonds but preserving the covalently imposed topography of the intramolecular arrangement of the DNA substrate; the other enzymes break up and/or restore covalent bonds rendering the covalent intramolecular substrate rearrangements. These basic differences might suggest the existence of a seventh class of enzymes in addition to the other six classes considered so far. In the same context, the dynamics of DNA nucleosomal coiling [7, 8] and nucleosomal supercoiling [9] during the cell cycle and gene function [22], as well as the dynamics of polycationic polionosomal condensation described here, would outline the catalytic topoisomerase-like behaviour of histones and protamine, constituting together this assumed seventh class of enzyme-like factors, which might be termed either *stereozymes*, or *stereases (conformases)*. The time span, the irregular velocity and the complexity of the sterease — macromolecular interactions make them difficult to be integrated within the limits of classical laws [16] governing enzyme reaction kinetics.

The ability of sterease activities to preserve the covalent intramolecular topography of their macromolecular substrates would achieve a unique function, that of contributing to the conservation of the genetic information, in spite of the considerable DNA changes required by replication, transcription, repairing, recombination, genetic transfer, and cell division. This unique function would assimilate stereases to other constitutive factors deeply involved in the correct informational flow of gene expression, such as chromosomal nonhistone proteins [22] different factors (IFs, EFs, RFs) of protein synthesis [23, 24], calmodulin and hormone receptors [25]. This would also lend support to the proposal of the separate class of stereases. Among other common features that arise from the cited literature, one may mention: many factors — considered as stereases — are energetically dependent of ATP-ase and/or GTP-ase activities, are frequently phosphorilated, are involved in the regulation of gene expression at its different biochemical levels, and always have informational macromolecules as interactive substrates. However, if we accept the substrate covalence unchanging DNA topoisomerases as unwinding and/or relaxing enzymes, it would be apparently logical also to accept this supposed stereases class, with its different components and its distinct basic characteristics pointed out above. Evolutionarily, some enzymes engaged in modulation, inducing covalent substrate modifications, would have structurally fixed sterease-like factors as regulatory subunits such as might have been the case of *e.g.*, aspartate carbamoyl transferase [26].

4. Ca²⁺ effect on polycation bacteriolysis; transient conformational changes of the murameres involved in a model of ectobiological exchanges and DNA up-taking

At the end of incubation interval, the control polycation free Ca^{2+} samples in the *E. coli* series had an $OD_{340} = 0.30$ and 5×10^7 CFU/ml. The control Ca^{2+} free samples not kept at 0 °C of the polylysine and polyarginine series had $OD_{340} = 0.08$ and $OD_{340} = 0.04$, respectively, with survival values of 7×10^4 CFU/ml and 3×10^4 CFU/ml, respectively.

Ca^{2+} addition to polylysine samples of *E. coli* strain PRC 399 induced a high rescue from bacteriolysis, OD values being constantly close to 0.30. The survival values of the same samples ranged between 2.5×10^7 CFU/ml and 3.3×10^7 CFU/ml; the mean percentage survival value was of 58, being higher by about three orders of magnitude than that found in the control Ca^{2+} free lytic samples of polylysine. As illustrated by OD values and CFU scoring, Ca^{2+} samples of the polylysine series display a discrepancy between unchanged turbidities and survival values, pointing to an important lytic free bactericidal action of polylysine. Although the 0 °C treatment accompanying Ca^{2+} addition had no obvious influence on the Ca^{2+} rescue, exposure to low temperature alone, for 30 min before and 30 min after polylysine addition, resulted in a significant percentage survival value of 5.5.

In the polyarginine samples of *E. coli*, Ca^{2+} and 0 °C rescues were lower (average survival values of 18% and 1.7%, respectively); however, the significance was still high as compared with the survival value of about 0.05% provided by Ca^{2+} free control samples of polyarginine not kept at 0 °C. OD values were under 0.10 in all the polyarginine samples being concordant with the survival values mentioned above.

The values of Ca^{2+} rescue in *B. subtilis* were much lower as compared with the *E. coli* series. Survival values reached only 0.1% for both polyarginine and polylysine series. This percentage may be, however, considered as significant if compared with the survival in the control Ca^{2+} free samples where, the values ranged between zero and 0.01%. 0 °C treatment accompanying Ca^{2+} addition had no significant influence, and 0 °C treatment alone gave no rescue in the *B. subtilis* series. As expected, the limited Ca^{2+} rescue was not discernible by OD

estimation since all the polycation samples of *B. subtilis* were cleared up having an $OD_{340} = 0.01 - 0.06$ at the end of the incubation period.

The evident influence of Ca^{2+} on the polycation action, mostly displayed in *E. coli* cells, may be due to their common sites of cell surface interactions. Ca^{2+} has multiple binding affinities for different envelope constituents [27] such as peptidoglycans, lipopolysaccharides, membrane proteins, and its action may be mediated in *E. coli* cells by a calmodulin-like factor [28]. In this context, Ca^{2+} inhibition of polycationic bacteriolytic and lytic free bactericidal effects would be explained by a possible competitivity exerted by bivalent cation action. Ca^{2+} ions act on the same mural sites as the polycation does, but having no condensation abilities, they induce nonlytic conformational changes masking polycationic affinity groups, and interfering with the pycnotic effect of the polycations.

The protective effect against the polycation action of 0 °C treatment alone observed in *E. coli* cells and not in *B. subtilis* cells, suggests that the wall lipid moiety, which is rich in Gram negative and poor in Gram positive bacteria [11], may be involved in polycation − wall interactions. The latter would be inhibited to some extent by the membrane lipid phase transitions previously described [29, 30] as caused by low temperature. 0 °C treatment inhibition can not be explained by the temperature dependent decrease of polycation − cell interactions, since the OD and survival values of the control *E. coli* samples not exposed to 0 °C, shake incubated at 37 °C for 15 min just after addition of the polycation, were as low as in the control samples kept at 25 °C.

The higher Ca^{2+} interference with polycationic cell lysis in *E. coli*, the increase of bacterial membrane permeability by Ca^{2+} treatment [31] and its wide use in inducing competence for DNA uptake in gene manipulation transformation (*e.g.*, [32, 33]), as well as the apparent discontinuous subunitary structure of the wall core inferred from multizonal polycation condensation, would lead to the suggestion of a model of active DNA uptake, possibly also involved in other ectobiological functions.

Assuming a murameric structure of the murein sacklike ensemble of the *E. coli* cell wall, direct and/or calmodulin − mediated interaction of Ca^{2+} with muramere constituents, would cause murameric conformational transitions of the type plate state $\rightleftharpoons$ globular state. The globular forms, possibly similar

to condensed polionosomes (Fig. 2), would delimitate pits. Their sides would be formed by complementary murameric surfaces, and their bottom by the cytoplasmic membrane. At least the pit sides may have some free cationic groups, e.g., amino groups of lysyl residues, interactive with DNA molecules and thereby constituting DNA binding sites. Due to its reversible character, the globular muramere form would undergo reverse conformational changes to the plate state; it will close tandemly the DNA binding site pit and thereby trapping possibly bound DNA molecules. The heat pulse enabling the cells to take up R-DNA [34] would quicken the DNA trapping closure of the pit sites. The assumed structure of DNA − trapping pit sites would explain why about 75% of the irreversibly bound DNA in Ca^{2+} stimulated recipient cells was associated with the cytoplasmic membrane fraction [27]. The intermurameric trapping of DNA molecules may bring them in intimate interactions with the cytoplasmic membrane zone forming the pit bottom. Murameric conformational changes would induce corresponding transient modifications in the outer membrane, intimately associated with the peptidoglycan core in a joined cell wall structure [35]. The same subunit globularization would cause, at least in the pits areas, local alterations in the tensile forces of the cytoplasmic membrane, making it more permeable. Over the entire bacterial envelope, such trapping pits would be successivley opened and closed, otherwise being too deleterious for cell survival; the forced action of Ca^{2+} in excess would accordingly increase the number of pits per cell, which may account for the important decrease in the survival values of the E. coli population induced to competence by Ca^{2+} [31, 34].

The model of DNA trapping pits may also account for DNA uptake in chromosomal and plasmid transformation [36] and transfection [37], at least in B. subtilis. The competence factors isolated from competent bacteria [38] induced under poor nutrient conditions [39], would cause globular transitions in the muramere conformation, forming tandemly associated interglobular pits, which act as DNA uptaking traps. The reverse change of the murameres to the plate state would close the pits catching DNA molecules previously attached on the inner surface of the trapping pits. Trapped DNA joined to the cytoplasmic membrane corresponding to the pit bottom, would be exposed to the peripheric processing described previously [40] and further internalized, possibly by a pinocytosis-like mechanism described in eukaryotes [14]. Polycation deceleration of muramere conformational changes involved in DNA pit trapping may account for the inhibition of genetic transformation by sublethal protamine concentrations [41]. The limited Ca^{2+} rescue of B. subtilis cells exposed to polycation bacteriolysis suggests the ability of the divalent cation to induce the same conformational changes resulting in intermurameric pits and agree with the findings that Ca^{2+} enhance the efficiency of plasmid transformation in B. subtilis [36]. So far there is little knowledge of how exogenous DNA is uptaken and transported through the envelope ensemble [27, 42]; the model of an active uptake system assumed here seems to provide a satisfactory understanding of this process.

The asynchronous formation of the pits and the fact that the globular state is more transient than the plate steady state of the murameres, would allow the cell wall to fulfill its other functions. The conformational changes of the murameres causing wall pits, would lead to the direct zonal contact of the cytoplasmic membrane with environmental constituents, which seems to be the single mechanism of macromolecular transenvelope transport, facilitating the activities of transmembrane transport-transfer-symport systems [43] of the prokaryotes. In other words, there would be a muramere "breathing" favouring the intimate interactions between cytoplasmic membrane components and environmental factors, thereby actively facilitating the functions of substrate-specific membrane proteins, i.e., porters, carriers, permeases, porines [11, 44]. Ectobiological signals (e.g., Ca^{2+}) inducing asynchronously the fast reverting globular states make them difficult if not impossible to be structurally revealed. Due to their pycnotic abilities, polycations harmfully stabilizing and amplifying the murameric globular states to stable polionosomes, reveal the existence of functional murameres otherwise unnoticed as long as they operate normally.

The results of the present work and the theoretic considerations seem to round off an active uptaking function of the cell wall corresponding to a structural system. The uptaking system would consist of: conformation subunits − the murameres; ectobiological factors inducing muramere globular transitions, e.g., Ca^{2+}; murameric reversible transitions from the plate steady state to the fast reverting globular state

tandemly accompanied by intermurameric trapping pits. This uptaking system appears to be functionally synergic and concordant with specific transfer-transport-symport systems already described [43] and seems to complete the transenvelope molecular crossing with its mechanism required for macro-molecular endocytosis in prokaryotes. Evolutionarily, it may be considered as the most rudimentary system of catching and internalizing exocellular molecules, superposed at this level of speciation with

the DNA trapping apparatus under the conditions of a possible genetic transfer. The intermurameric pits would be some errant cytostome-like structures, appearing here and there on the bacterial surface, not having as yet a defined site as, *e.g.*, the cyto-stome of the eukaryotic *Paramoecium* species cell.

Acknowledgement

[1] S. Antohi and Al. Popescu, Z. Naturforsch. **34 c,** 1144–1150 (1979).
[2] Al. Popescu, S. Antohi, S. Trasculescu, A. Aurescu, N. Manolescu, and V. Ciocnitu, Z. Naturforsch. **35 c,** 805–810 (1980).
[3] A. G. Marr, in The Bacteria (I.G. Gunsalus and R. Y. Stanier, eds.) **Vol. 1,** pp 443–468, Academic Press, New York and London 1960.
[4] S. E. Luria, in The Bacteria (I. G. Gunsalus and R. Y. Stanier, eds.) **Vol. 1,** pp 1–34, Academic Press, New York and London 1960.
[5] E. M. Lederberg, Plasmid Reference Center, Dept. of Medical Microbiology, Stanford University, Stanford, California.
[6] M. Renz, P. Nehls, and J. Hozier, Proc. Natl. Acad. Sci. USA, **74,** 1879–1883 (1977).
[7] I. Isenberg, Ann. Rev. Biochem. **48,** 159–191 (1979).
[8] D. W. Martin, Jr., in Review of Physiological Chemistry (H. A. Harper et al., eds.), pp. 135–146, Lange Medical Publications, Los Altos 1979.
[9] J. Allan, P. G. Hartman, C. Crane-Robinson, and F. X. Aviles, Nature **288,** 675–679 (1980).
[10] J. W. Costerton, J. M. Ingram, and K.-J. Cheng, Bacteriol. Rev. **38,** 87–110 (1974).
[11] G. Drews, in Progress in Botany (H. Ellenberg et al. eds.) **42,** 16–24, Springer Verlag, Berlin Heidelberg New York 1980.
[12] G. Lancelot, R. Mayer, and C. Hélène, Biochim. Biophys. Acta **564,** 181–190 (1979).
[13] G. E. Schulz and R. H. Schirmer, in Principles of Protein Structure, p. 206, Springer Verlag, New York Heidelberg Berlin 1979.
[14] S. C. Silverstein, R. M. Steinman, and Z. A. Cohn, Ann. Rev. Biochem. **46,** 669–722 (1977).
[15] J. L. Strominger, Johns Hopkins Med. J. **133,** 63–81 (1973).
[16] A. L. Lehninger, in Biochemistry, 2nd Edition, p. 268, Worth Publishers, Inc. New York 1975.
[17] R. J. Salton, Ann. Rev. Microbiol. **21,** 417–442 (1967).
[18] J. L. Strominger, K. Izaki, M. Matsuhashi, and D. J. Tipper, Fed. Proc. **29,** 9 (1967).
[19] P. O. Brown, C. L. Peebles, and N. R. Cozzarelly, Proc. Natl. Acad. Sci. USA, **76,** 6110–6114 (1979).
[20] H. F. Kung, C. Spears, and C. Weissbach, Arch. Biochem. Biophys. **174,** 100–104 (1976).
[21] Y. J. Topper, in The Enzymes (P. D. Boyer, H. Lardy & K. Myrbäck, eds.) **vol. 5,** pp. 413–428 Academic Press New York 1961.
[22] K. Mita, M. Zama, S. Ichimura, and K. Hamana, Biochem. Biophys. Res. Comm. **98,** 330–336 (1981).
[23] T. Staehelin, H. Trachsel, B. Erni, A. Boschetti, and M. H. Schreier, in Cell Differentiation in Micro-organisms, Plants and Animals (L. Nover & K.

Mothes, eds.) pp. 183–208, VEB Gustav Fischer Verlag, Jena 1977.
[24] S. Ochoa and C. de Haro, Ann. Rev. Biochem. **48,** 549–580 (1979).
[25] A. R. Means and J. R. Dedman, Nature **285,** 73–77 (1980).
[26] H. L. Monaco, J. L. Crawford, and W. L. Lipscomb, Proc. Natl. Acad. Sci. USA, **75,** 5276–5280 (1978).
[27] A. Sabelnikov and I. V. Domaradsky, Molec. Gen. Genet. **172,** 313–317 (1979).
[28] Y. Iwasa, K. Yonemitsu, K. Matsui, K. Fukunaga, and E. Miyamoto, Biochem. Biophys. Res. Comm. **98,** 656–660 (1981).
[29] A. J. Verkleij, P. H. J. Ververgaert, L. L. M. Van Deenen, and P. F. Elbers, Biochim. Biophys. Acta, **288,** 326–332 (1972).
[30] P. Overath, M. Brenner, T. Gulik-Krzwiki, E. Schechter, and L. Letelier, Biochim. Biophys. Acta **389,** 358–369 (1975).
[31] M. Mandel and A. Higa, J. Mol. Biol. **53,** 159–162 (1970).
[32] S. N. Cohen, A. C. Y. Chang, H. W. Boyer, and R. D. Helling, Proc. Natl. Acad. Sci. USA, **70,** 3240–3244 (1973).
[33] J. Collins, in Current Topics in Microbiol. & Immunol. (W. Arber et al., eds.) **78,** 121–170, Springer Verlag, Heidelberg Berlin New York 1977.
[34] S. N. Cohen, A. C. Y. Chang, and L. Hsu, Proc. Natl. Acad. Sci. USA, **69,** 2110–2114 (1972).
[35] J. Weckesser, G. Drews, and H. Mayer, Ann. Rev. Microbiol. **33,** 215–239 (1979).
[36] S. Contente and D. Dubnau, Molec. Gen. Genet. **167,** 251–258 (1979).
[37] T. A. Trautner and H. C. Spatz, in Current Topics in Microbiol. & Immunol. (W. Arber et al., eds.) **62,** 61–88, Springer Verlag, Berlin 1973.
[38] A. Akrigg, S. R. Ayad, and G. R. Barker, Biochem. Biophys. Res. Comm. **28,** 1062–1067 (1967).
[39] C. Anagnostopoulos and C. Spizizen, J. Bacteriol. **81,** 741–746 (1961).
[40] D. Dubnau and C. Cirigliano, J. Mol. Biol. **64,** 9–29 (1972).
[41] S. Antohi and Al. Popescu, Molec. Gen. Genet. **170,** 345–349 (1979).
[42] R. W. Old and S. B. Primrose, in Principles of Gene Manipulation. An Introduction to Genetic Engineering. Blackwell Scientific Publications, Oxford London Edinburgh Boston Melbourne 1980.
[43] R. Harrison and G. G. Lunt, in Biological Membranes, Second Edition pp. 166–194, Blackie, Glasgow and London 1980.
[44] E. Padan, L. Patel, and H. R. Kaback, Proc. Natl. Acad. Sci. USA **76,** 6221–6225 (1979).