J. Membrane Biol. 100, 229–242 (1987)

The Journal of
Membrane Biology

Polymorphic Behavior of Gram-Negative Bacteria Membranes

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Summary. Freeze-fracture and ultrathin section electron microscopy as well as ^{31}P-NMR spectroscopy and light scattering of *Escherichia coli* and *Pseudomonas putida* cells under conditions promoting the ability of cells to take up exogenous DNA's (high concentrations of divalent cations and a specific temperature regime) reveal the extensive polymorphic changes and the formation of various structural defects in cellular membranes. Polymorphic changes occur during the heat shock at 42 to 44°C of the cells preincubated at 0°C in the presence of high concentration of Ca^{2+} or Ba^{2+} cations and include the formation of various vesicle- and tube-like structures, intermembrane and intercellular contacts followed by membrane fusion and sometimes even by cell fusion. The results obtained suggest the occurrence of phospholipid-enriched zones in the outer leaflet of *E. coli* outer membrane. This suggestion is verified and confirmed with the help of phospholipase C, a specific phospholipid binding and digesting enzyme. The presented experimental evidence directly supports the suggestion of Ahkong et al. (*Nature* **253**:194–195, 1975) on the identity of the mechanisms of membrane contact formation and membrane fusion in model and cellular membranes. The biological relevance of the polymorphic changes observed is shortly discussed.

Key Words bacterial membranes · polymorphism *in situ* · divalent cations · DNA uptake

Introduction

Lately the functional role of cell membrane lipids has been extensively revised (Cullis & De Kruijff, 1979; Verkleij, 1984; Cullis et al., 1985). In view of polymorphic phase transitions observed in different model membranes, possible functional role of these phenomena in cellular membranes in vivo has been suggested. Polymorphic changes are thought to be intimately involved in such processes of cell physiology as transmembrane movement of certain intracellular and extracellular compounds, intermembrane and intercellular contact formation, and membrane fusion phenomena (Cullis & de Kruijff, 1979; Pinto da Silva & Kachar, 1980; Cullis et al., 1985).

During the past few years the lipid polymorphism (Rand & Sengupta, 1972; Miller, 1980; Hui et al., 1981; Borovjagin, Vergara & McIntosh, 1982; Markin, Kozlov & Borovjagin, 1984; Verkleij, 1984; Düzgünes, 1985; Vasilenko, Tarahovsky & Borovjagin, 1986) and intermembrane contact formation mechanisms (Papahadjopoulos, 1968; Ahkong et al., 1975; Lucy, 1984; Düzgünes et al., 1985; Düzgünes, 1985; Vasilenko et al., 1986) in model membrane systems have been extensively investigated.

However, the question of biological relevance of the phenomena observed in model membranes is still being debated since, until now, the polymorphic changes have not been observed in biomembranes *in situ*.

There is some evidence concerning the polymorphic transitions in isolated biological membranes such as frog rod outer membrane segment (Corless & Costello, 1981), membranes of chloroplasts (Murphy, 1982), and of mitochondria (Van Venetié & Verkleij, 1982). However, no functional role of the observed structures has been elucidated or suggested. Rather they were considered as artefacts of isolation and preparation.

Recently, from the results of ^{31}P-NMR spectroscopy of *E. coli* cells pretreated with high concentrations of some divalent cations (Ca^{2+}) at 0°C and then heat shocked at 42°C, the formation of nonbilayer lipid structures in cellular membranes in vivo has been inferred (Sabelnikov et al., 1985). Furthermore, the functional role of these polymorphic changes has been suggested: their direct involvement in exogenous DNA uptake by the cells observed under the employed assay conditions.

In view of the importance of both the above suggestions we have undertaken a more detailed and thorough study of structural changes of bacterial membranes and of their relevance to transmembrane DNA movement. The results of this study are presented in this paper.

Materials and Methods

The cells of *E. coli* K12 P400 (wild strain) and P530 (omp C, F derivative) strains (Schweizer et al., 1976) and those of other taxonomically unrelated bacteria *Pseudomonas putida* BS250 were grown, harvested and treated with divalent cations as described earlier (Sabelnikov & Domaradsky, 1981; Tarahovsky et al., 1986) with the following minor modifications: *P. putida* cells were grown at 30°C rather than at 37°C. After 24-hr incubation of cells with 100 to 150 mM of $CaCl_2$, $BaCl_2$ or $MgCl_2$ solutions, at 0°C the samples were heat shocked for various periods of time from 3 min to 2.5 hr.

Light Scattering

Light-scattering experiments were performed on a Jobin-Iv-on 3CD spectrofluorometer. The intensity of the scattered light was measured at a 90° angle in 1-cm cuvettes, when both monochromators were set at 600 or 650 nm.

^{31}P-NMR Spectra

^{31}P-NMR spectra were measured on a Bruker WM 250 high resolution spectrometer essentially as described earlier (Sabelnikov et al., 1985).

Freeze Fracture

Freeze-fracture experiments were performed by the techniques described by Costello and Corless (1978). Cell samples, with or without cryoprotectants (15% glycerol), were placed between the two copper strips and quenched from the temperatures specified above by plunging them into liquid propane (−190°C) at cooling rates in excess of ±8.000°C/sec (Costello, 1980). The frozen samples were inserted into a hinged double replica device adapted for use on modified JEE-4C (JEOL, Japan) freeze-fracture unit. Up to 10 samples were simultaneously fractured at −150°C and about 10^{-7} torr. Some samples were fixed with 1% glutaraldehyde (cocadilate buffer 100 mM, pH 7.4, 1 hr), washed three times by bidistilled water, precipitated (500 rpm, 20 min) and freeze etched for 1 to 5 min at 99°C. Fractured and etched samples were replicated with carbon-platinum from a 45°C angle and with carbon from a 90°C angle by means of electron guns.

The replicas were cleaned in Clorox®, washed in water and picked up on uncoated 400-mesh electron-microscope grids.

Thin Sectioning

For thin sectioning different cell samples were fixed with 1% glutaraldehyde (cocadilate buffer 0.1 M; pH 7.4, 1 to 2 hr) and postfixed with 1% OsO_4 (pH 7.2, 1 hr at 4°C). The specimens were then dehydrated in an ethanol series, and embedded in Epoxy resin (Epon 812). Thin sections were cut on LKB Ultratom III, stained with 2% uranyl acetate and 0.5% lead citrate.

All samples were examined in an electron-microscope JEM-100C (Japan, JEOL) equipped with an anticontamination device cooled with liquid nitrogen.

Transformation Assays

Transformation assays with pALR2 plasmid DNA bearing ampicillin resistance were performed on cells treated as described above by standard methods (Sabelnikov & Domaradsky, 1981; Sabelnikov, Gordienko & Ilyashenko, 1984). The cell viability was checked by the routine dilution and plating technique (Sabelnikov & Domaradsky, 1981). To escape possible artefacts caused by the tight aggregation of some cells in the presence of Ca^{2+} and Ba^{2+} during the heat shock (*see text*) the cell samples, before the determination of cell viability, were diluted six- to 10-fold with L-broth and incubated with aeration at 37°C for 30 to 40 min.

Phospholipase C Treatments

Commercially available phospholipase C from *Bacillus cereus* (USSR firm "FERMENT") was used in all the experiments. The purity of the enzyme has been checked by polyacrilamide gel electrophoresis. To detect the occurrence of phospholipids in the outer leaflet of the outer membranes of *E. coli*, the accessibility of surface phospholipids to exogenously added phospholipase has been investigated according to (Duckworth et al., 1974; Van Alphen et al., 1977). The cells treated with 0.1 M $CaCl_2$ overnight at 0°C were spun down and resuspended in 0.01 M $CaCl_2$ at room temperature. The enzyme was then added to the suspension, and the mixture was incubated with gentle shaking for 2 to 3 hr at 37°C. At the end of the incubation period EDTA (10 mM final concentration) and egg-white lysosyme (Serva, 200 μg per ml, final concentration) were added and the lipids were extracted with methanol-chloroform according to Bligh and Dyer (1954). The products of hydrolysis were analyzed by thin-layer chromatography on silica gel as described by Duckworth et al. (1974).

To study the effect of phospholipase C on DNA uptake the enzyme was added to cells in the presence of 0.01 M $CaCl_2$ prior to exogenous DNA, and the cell-enzyme mixture without DNA was incubated at 0°C for various time intervals from 15 min to 12 to 14 hr. After that the cells were either spun down and resuspended in 0.1 M $CaCl_2$ or the mixture was made 0.1 M $CaCl_2$ with 1 M $CaCl_2$ and exogenous DNA was added. The rest of the protocol was according to Sabelnikov and Domaradsky (1981). In some experiments the enzyme was directly added to cells in 0.1 M $CaCl_2$ at 0°C prior to or after the addition of exogenous DNA. In samples where the enzyme was added to cells, already incubated with exogenous DNA at 0°C for 30 min, the cell-DNA-enzyme mixture was immediately heat shocked and treated as above.

Results and Discussion

^{31}P-NMR data (Sabelnikov et al., 1985) have suggested that polymorphic changes (if any) in bacterial membranes occur at 37 to 42°C in cells preincubated at 0 to 2°C in the presence of high concentrations of divalent cations (Ca^{2+}, Ba^{2+}). Therefore the structural changes of cellular membranes were investigated during these sequential stages of treat-

ment. The addition of divalent cations to bacterial suspensions at 0°C results in profound and reversible cell aggregation as evidenced by the light-scattering experiments (Fig. 1). The effect is due most likely to the neutralization of negative charges, dehydration of cell surfaces, and possibly to cation-induced cell fimbriae interaction as seen on the micrograph of a freeze-etched sample (Fig. 2*A*, arrowheads).[1] Electron-microscopy results reveal the close approach of cells without the formation of intercellular contacts (*not shown*).

In addition, as evidenced by the results of electron microscopy, the preincubation of cells with cations (Ca^{2+}, Ba^{2+}) at 0°C provides lateral phase separation and segregation of components in both cell envelope membranes, which results in the formation of extensive smooth particle-denuded areas seen on fracture faces of both the outer and cytoplasmic membranes (Fig. 2*A*,*B* and *B'*, arrowheads). This phenomenon does not depend on whether the samples contain glycerol as a cryoprotector during quenching. Intermembrane particles' (IMP's) segregation in outer membranes is further manifested by the infrequent releasing of membrane vesicles, or blebs from the cell surface which exhibit fracture faces poorly decorated by IMP's (Fig. 2*C*, *D*, arrowheads).

It is worthwhile to note that in some cells, particularly those of *Pseudomonas putida*, the envelope membranes in these conditions exhibit multilayer structure practically devoid of IMP's (Fig. 2*E*, asterisks).

As a result of these changes multiple defects appear in the bilayer structure of the outer membranes which may account for the formation of nonspecific outer membrane permeability for various compounds of different chemical nature and molecular mass, such as membranotropic drugs and metabolic inhibitors, antibiotics, periplasmic binding proteins and periplasmically located enzymes (for a review, *see* Brass, 1986).

Subsequent heat shock (3 to 15 min at 42 to 44°C) of the cells preincubated with calcium or barium cations at 0°C produces several characteristic effects. Firstly, it greatly enhances the formation and release of vesicles or blebs from the outer membrane surface (Fig. 3*B*). Secondly, the heat shock promotes the formation of various polymorphic membrane structures, such as vesicles and tube-like (often multibilayer) structures (Fig. 3*A*, *B*, *B'*, *C* and *D*), and their tetragonal or hexagonal arrangements (Fig. 3*B'*). Thirdly, the heat shock initiates multiple cell-to-cell contacts. This might account for the partially irreversible aggregation of cells observed under these conditions in the light-scattering experiments (Fig. 1). These tightly bound aggregates, however, seem to dissociate if the cell suspension is diluted with L-broth and incubated with aeration at 37°C for 30 to 40 min (Sabelnikov, *unpublished results*).

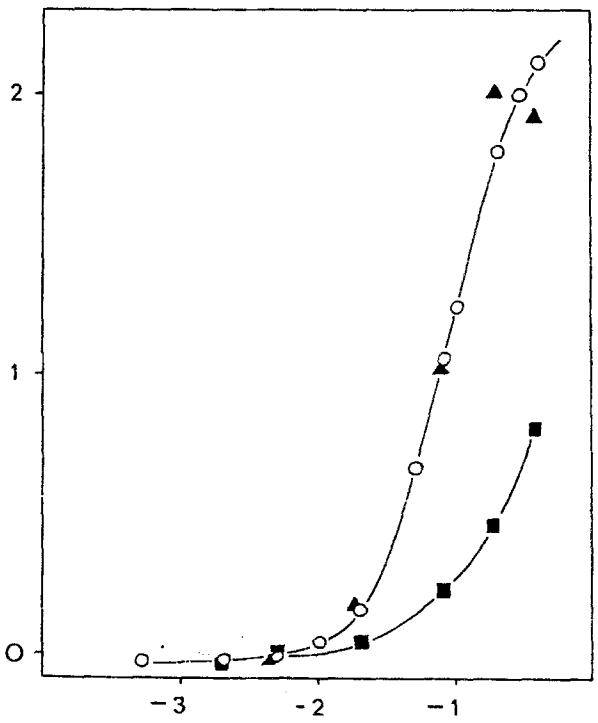


Fig. 1. Dependence of a 90° angle light scattering of the cell suspensions on the cation concentration. The results with Ca^{2+} are presented. (Similar results were obtained with other divalent cations.) ○—the cells of *E. coli* P400 (or P530), ▲—the cells of *P. putida* BS350; ■—the cells of *E. coli* P400 (○) washed twice with 0.005 M Tris-HCl, pH = 7.4. Abscissa is the concentration of Ca^{2+} (M) in logarithms, ordinate is the relative intensity of scattered light $\left(\frac{F_i - F_o}{F_o}\right)$

Cell-to-cell contacts are clearly seen both on thin sections (Figs. 4 and 5) and on the fracture faces (Figs. 5, 6 and 7). Figure 4 (*A*, *A'* and *B*, arrowheads) shows the formation of multiple contacts between the membranes of several adjoining cells. Large cell surface areas are involved in the process of contact formation. It is likely that the

[1] All Freeze-fracture electron micrographs are negative printed; large white arrowhead indicates the platinum deposition direction; Bars on all micrographs is 0.1 mM; *OM* and *CM* mark the outer and the cytoplasmic bacterial envelope membranes; *PF* and *EF* mark the hydrophobic faces of the internal and the external membrane halves, respectively.

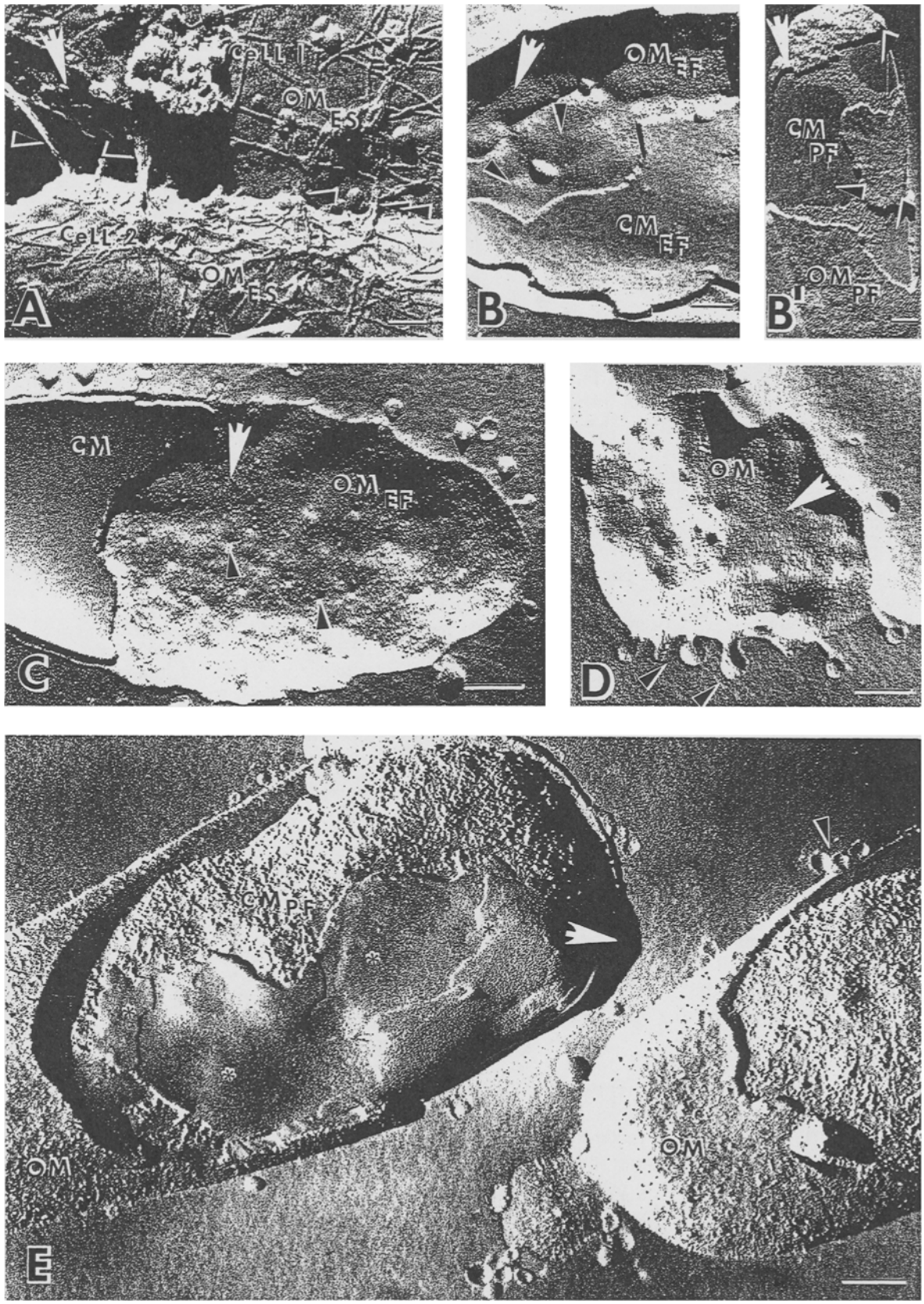


Fig. 2. Freeze etch (*A*) and freeze-fracture (*B*, *B'*, *C*, *D*, *E*) electron micrographs of *E. coli* P530 (*A*, *B*, *B'*, *C*) and *P. putida* BS250 (*D*, *E*) cells incubated at 0°C in the presence of 100 mM Ca^{2+} (or Ba^{2+}) during 60 min. All samples were quenched from +2°C. In *A*, the sample was etched for 4 min: numerous fimbriae are seen to be attached to the hydrophilic external surfaces of adjacent cells; some fimbriae may form bridges (single or double arrowheads) between the cells. Images *B* and *B'* show *EF* (*B*) and *PF* (*B'*) faces of envelope membranes bearing large areas devoid of IMP's, whereas images *C*, *D* and *E* illustrate the pinching off of outer membrane vesicles and blebs (arrowheads). In *E*, extensive multilamellar smooth faces are seen (asterisks), which are more frequently found between envelope membranes of *P. putida* cells

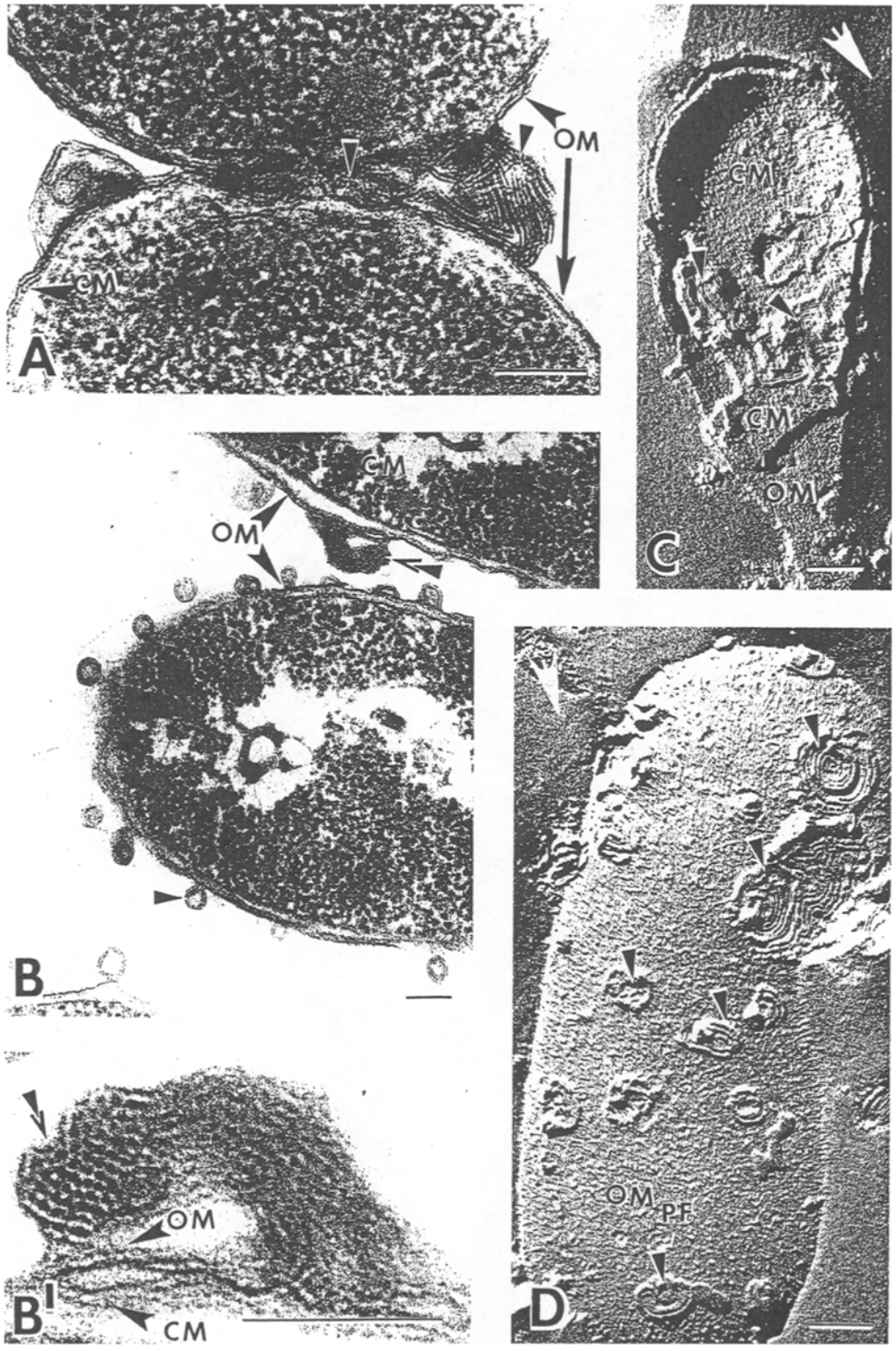


Fig. 3. Thin-section (*A*, *B*, *B'*) and freeze-fracture (*C*, *D*) images of *P. putida* BS250 (*A*, *C*) and *E. coli* P400 (*B*, *B'*, *D*) cells incubated at 0°C (overnight) in the presence of 100 mM Ca^{2+} and heat shocked for 5 to 15 min at 42°C. All samples are quenched from +42°C. The formation of multilamellar and/or tube-like structures composed of outer and cytoplasmic membranes of adjacent cells (*A*, arrowheads) or of envelope membranes of a single cell (*C*, *D*, arrowheads) are clearly seen in samples of both bacteria; sometimes polymorphic structures originate from outer membranes (*B*, *B'*, double arrowheads)

observed polymorphic structures may be involved in the formation of outer membrane contacts of adjoining cells (Figs. 4*C*; 5*B*, *C*; 6*A*, *B*; 7).

It must be emphasized that the polymorphic changes observed in cells during their incubation at elevated temperatures (37 to 44°C) occur only when the cells have been preincubated at 0°C in the presence of divalent cations.

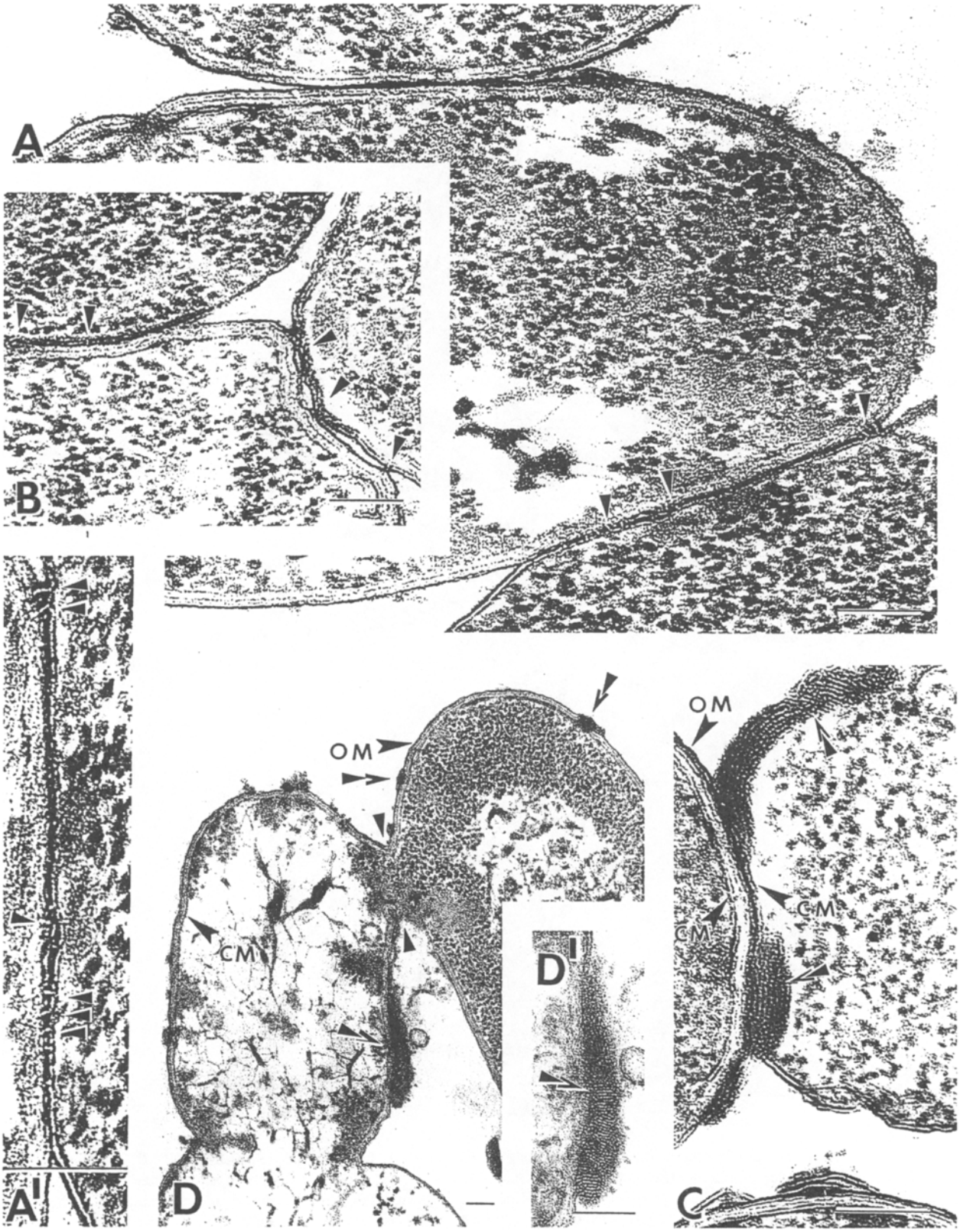


Fig. 4. Thin-section electron micrographs of *P. putida* (*A*, *A'*, *B*) and *E. coli* (*C*, *D*, *D'*) cells incubated at 0°C in the presence of 100 mM Ca^{2+} and heat shocked for 2 hr at 42°C. Numerous intermembrane contacts and pore structures (*A*, *A'*, *B*, arrowheads) are distinctly visible. In *C*, *D* and *D'*, the development of polymorphic structures leads to the fusion of envelope membranes (double arrowheads) as well as to local fusion of cells (the area marked between the two single arrowheads)

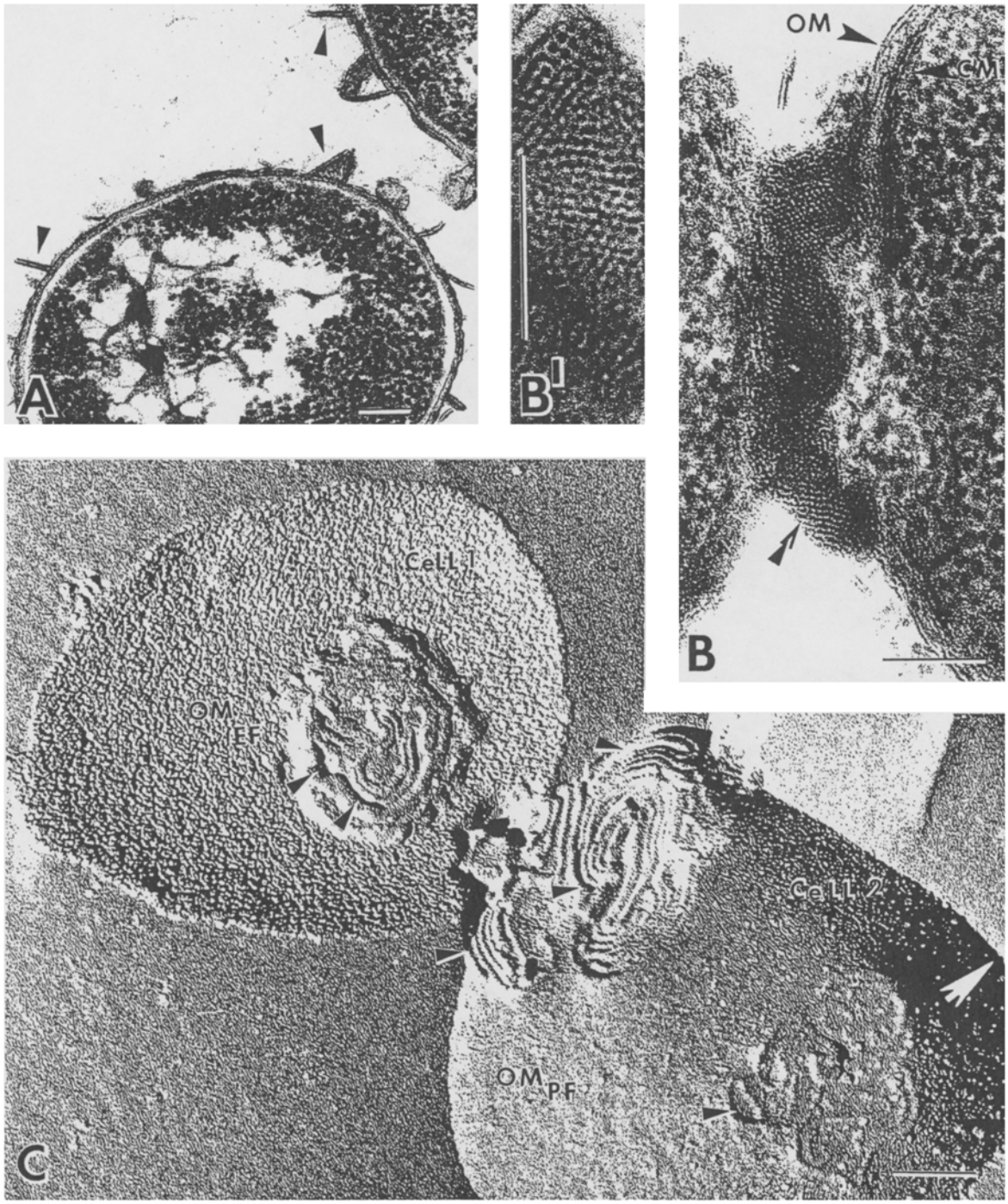
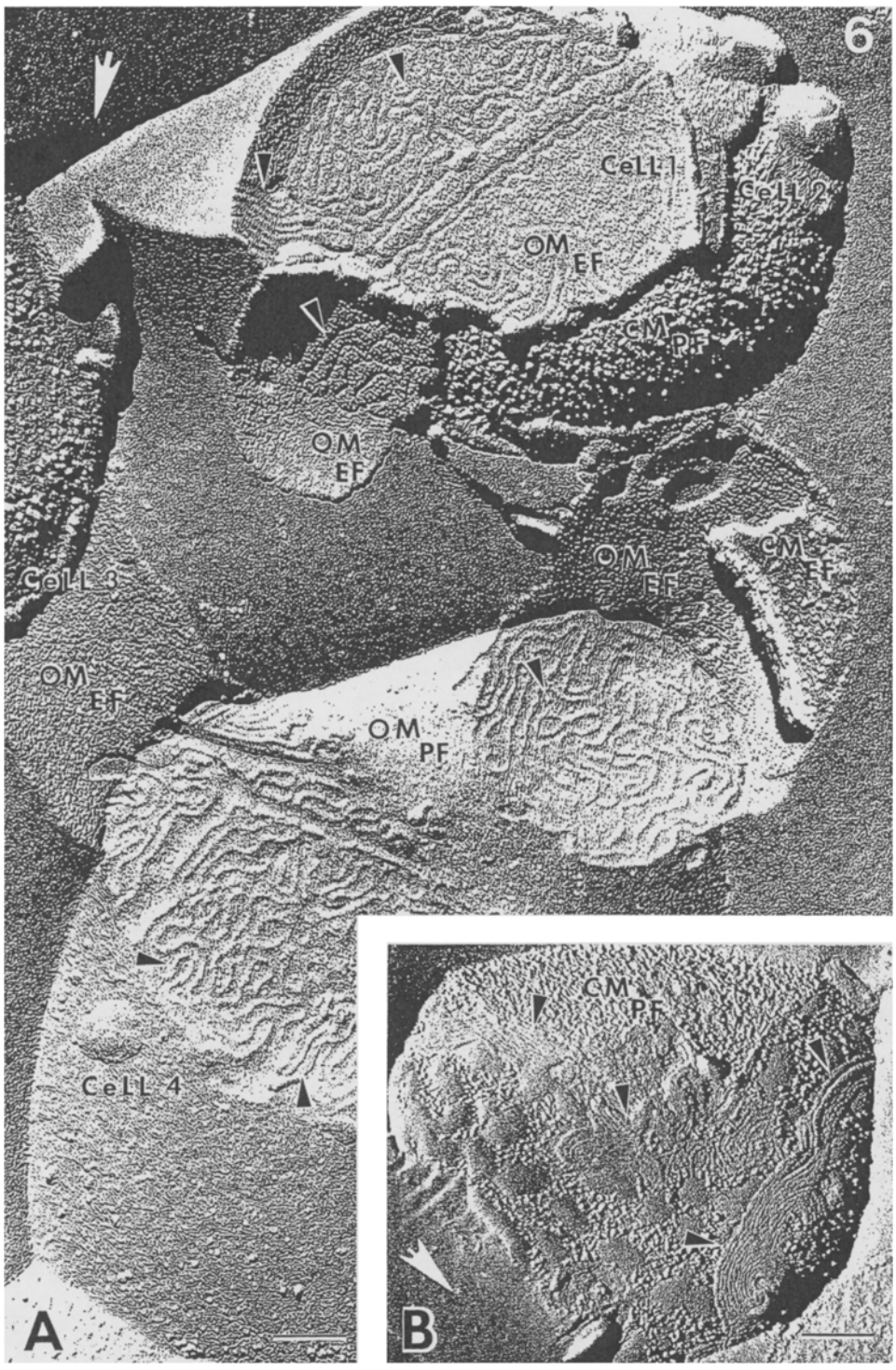


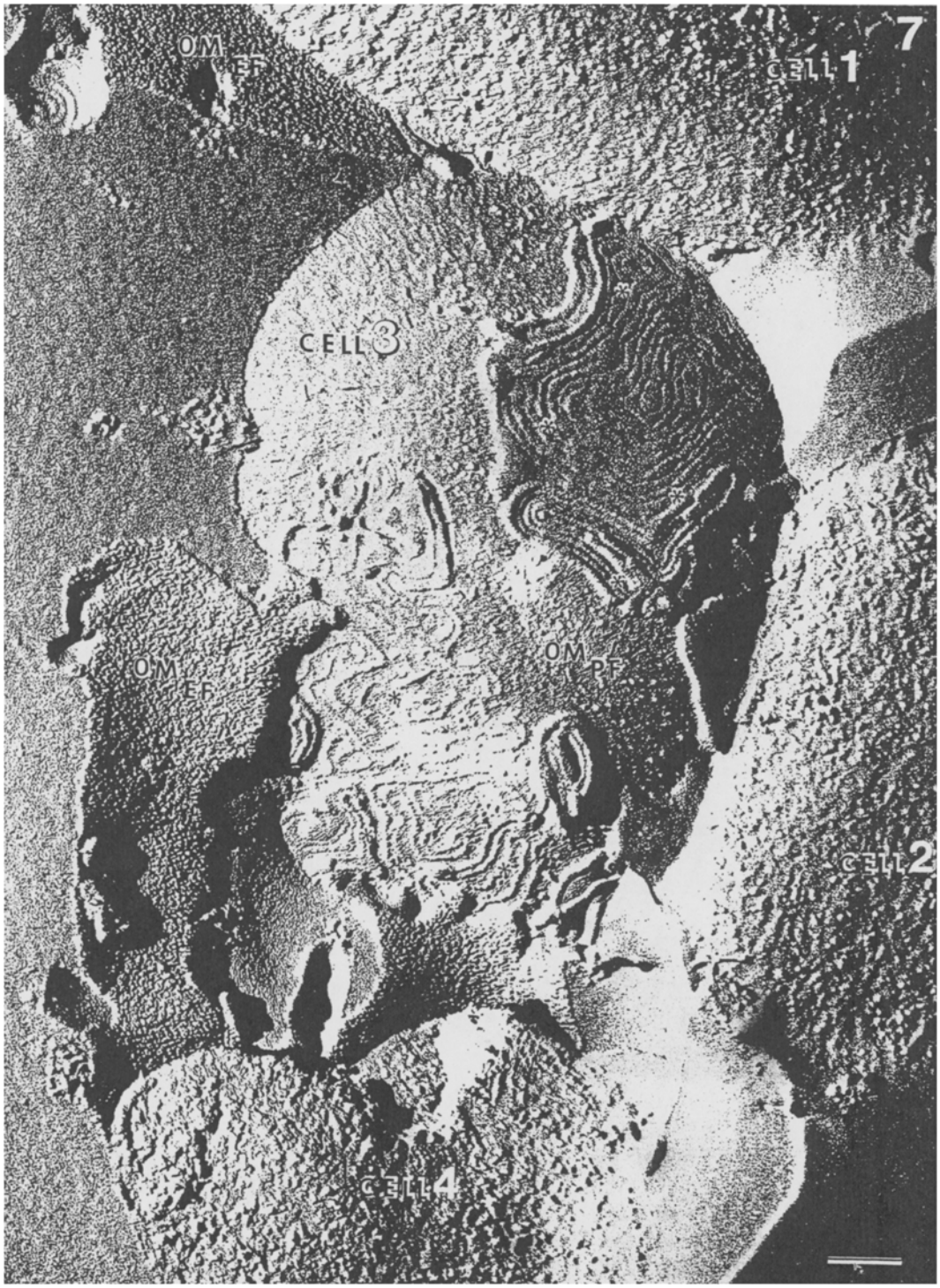
Fig. 5. Thin-section (*A*) *E. coli* P400 and (*B*, *B'*) *P. putida* and freeze-fracture (*C*) *E. coli* P400 electron micrographs of bacterial cells incubated at the same conditions as the samples in Fig. 4. The formation of tube-like (*A*, arrowheads) and polymorphic (*B*, *B'*, double arrowheads) as well as point, circular and linear contacts (*C*, arrowheads) formed as a result of the adjacent cells envelope membrane fusion are frequently observed

Prolongation of heat shock (up to 2 hr), results in the increase of irreversible cell aggregation and the enhancement of polymorphic changes in both cell envelope membranes. The process of membrane contact formation and of polymorphic changes now extends also to inner membranes. Contacts between outer and inner membranes (Figs. 3*C*; 4*A*, *A'* and *C*; 5*C*) of adjoining cells, poly-



Figs. 6 and 7. Freeze-fracture electron micrographs of *P. putida* (Fig. 6*A*, *B*) and *E. coli* (Fig. 7) cells incubated at the same conditions as in Figs. 4 and 5. Numerous point, circular and linear contacts (arrowheads and asterisks) between membrane of adjacent cells (Cells 1, 2, 3, 4) are visible. In 6(*B*) smooth areas on *PF* faces of cytoplasmic membrane and their polymorphic transition are clearly seen

morphic structures composed of both cell envelope membranes (Figs. 3*C*; 4*C*, *D'*; 5*B* and *C*; 6), as well as pore structure formation due to fusion of outer membranes (Fig. 4*A*, *A'*, *B*, arrowheads) are often seen on electron micrographs of ultrathin sections. Fracture faces reveal multiple point, linear, and circular contacts formed between the outer membranes of adjoining cells (Figs. 5, 6 and 7, arrow-



heads and asterisks) and, occasionally, between the outer and inner membranes (Figs. 3*C*, 5*C*, 6*B*).

After prolonged incubation at 37 to 42°C membrane contact formation starting in outer membranes of adjoining cells and then extending to their inner membranes may eventually result in local cell fusion (Fig. 4*D*) though with a very low frequency.

Since polymorphic changes in membranes during the heat shock are often associated with the formation of membrane contacts it is reasonable to suggest that they enter the chain of events leading to membrane fusion. The results of electron microscopy are summarized in the drawing in Fig. 8. (It is necessary to note, that intercellular DNA transloca-

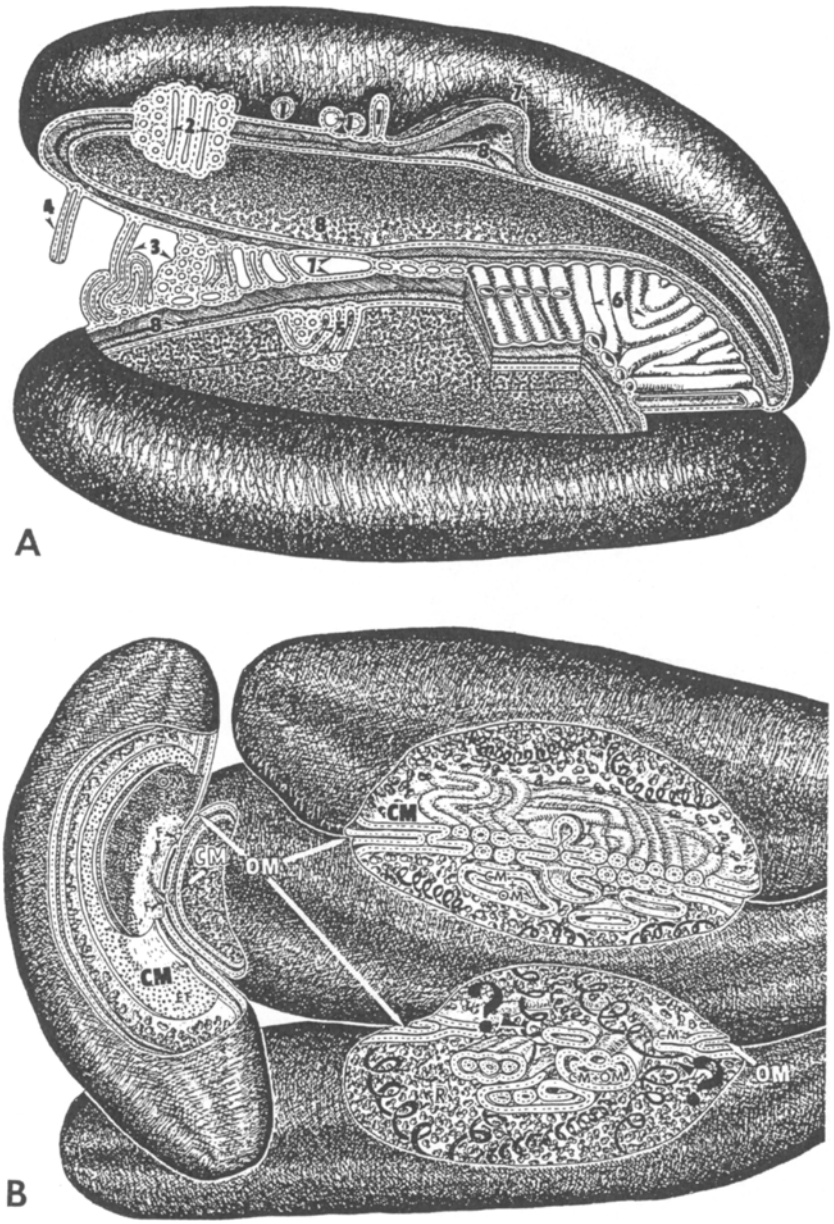


Fig. 8. Various membrane structure changes observed in freeze-fracture and thin-section micrographs of the samples incubated at the employed conditions are summarized. (*A*) 1—vesicles and blebs pinching off the outer membrane; 2, 3—polymorphic structures formed at the site of outer and cytoplasmic membrane fusion; 4—single tube-like structures originated from outer membrane and presumably formed by lypopolysaccharides; 5, 6—polymorphic structures formed in cytoplasmic membrane (5) and in the sites of tight contact of outer membranes of adjacent cells (6); 7, 8—outer and cytoplasmic membranes, respectively; (*B*) different stages of contact (*F*) formation between outer (*OM*) and cytoplasmic (*CM*) membranes as well as the sites of membrane fusion (*F*) are shown. Intracellular DNA (black coils) are also shown

tion between cells 3 and 4 depicted in Fig. 8*B* is purely speculative.)

There is striking similarity between the organization of point-like and linear contacts in cellular membranes observed here and the same structures usually observed at the initial stages of membrane contact formation in model membrane systems (*see*, for example, Ahkong et al., 1975; Vasilenko et al., 1986).

This strongly supports the suggestion of Ahkong et al. (1975) on the identity of the mechanisms of membrane contact formation and fusion in model and cellular membranes.

Based on the results obtained here we suggest that the polymorphic membrane behavior and its functional role *in situ* may be seriously considered only in relation to the cellular multimembrane systems (such as in chloroplasts, mitochondria, different cytoplasmic organelles, photoreceptor membrane complexes, etc.), or in cases when at least two or more cellular membranes come into close contact.

^{31}P-NMR spectra of cells during the heat shock characteristically reveal a new and distinctive signal with a maximum at about 20 ppm towards the low field from the signal of 85 percent H_3PO_4 (Fig. 9*A*, *B*; and Sabelnikov et al., 1985).

Several lines of evidence suggest that the changes in ^{31}P-NMR spectra are related to the formation of polymorphic structures revealed by electron microscopy here.

The enhancement of polymorphic changes with

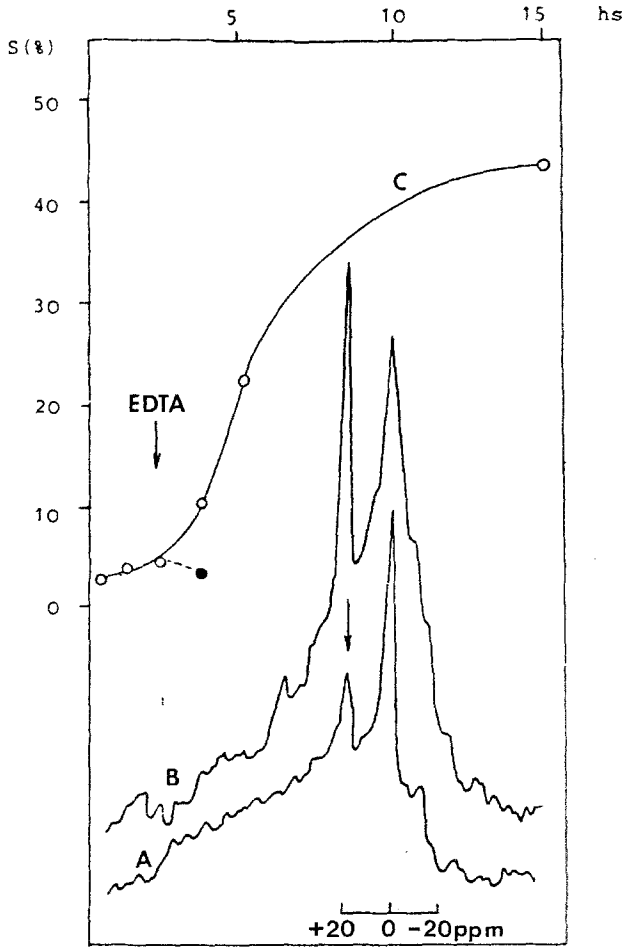


Fig. 9. Proton-decoupled ^{31}P-NMR spectra of *E. coli* cells, preincubated with $CaCl_2$ at 0°C (*A*) Spectrum recorded without saturation after 2-hr incubation at 42°C. (*B*) Difference spectrum between the saturated and unsaturated (*A*) spectra. (*C*) Kinetics of growth of intensity of the peak (as percent of the area under the spectrum integral curve) marked with the arrow. The latter also indicates the carrier frequency at which the saturation was applied

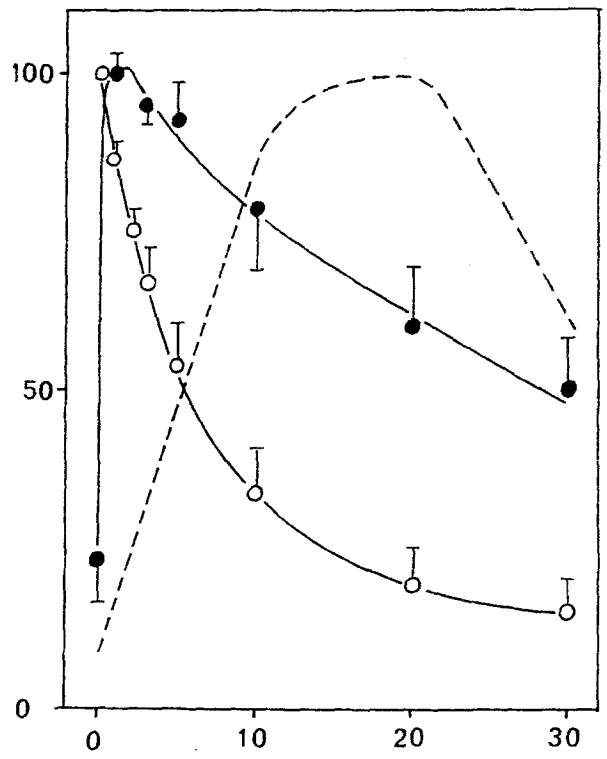


Fig. 10. Effect of the duration of the heat shock (at 42°C) on the cell viability (○) the number of Amp^r-transformants (the cells which took up plasmid DNA) (●), and the efficiency of DNA uptake (dotted line). Abscissa is the time in minutes; ordinate, the relative number of viable cells (≡ 1 at zero time) and of the number of transformants (maximal number of 10^5 per ml at 2 min ≡ 1)

time at 37 to 42°C parallels that of the intensity of the new signal (Fig. 9*C*). Besides, the enhancement of the vesicles' formation and their release from the cell surface (Figs. 2*C*, *D*; 3*B*) might be the cause of the enhancement of the isotropic resonance observed under these conditions by Sabelnikov et al. (1985). Furthermore, the results of saturation transfer experiments (Sabelnikov et al., 1985) have revealed the rapid exchange of phosphorus containing molecules between the newly formed (the new resonance) and the bilayer structures, and between the latter and the vesicle-like or micellar-like structures (isotropic signal). The appearance of all these suggested structures undergoing the exchange of their molecules readily falls within the sequence of events in the process of polymorphic membrane changes and the formation of membrane contacts inferred from the elecron-microscopy data (*see* Fig. 8).

Hence all these data unequivocally indicate that the new ^{31}P-NMR signal is generated by the observed polymorphic changes in membranes rather than by the conformational changes of phosphorus-containing molecules, e.g. phospholipid head groups or lypopolysaccharides (Thayer & Kohler, 1981; Noggle et al., 1982).

The crucial question concerning the evidence presented here is whether the observed polymorphic changes in cellular membranes are still compatible with the cell viability. Polymorphic changes are initiated by a heat shock and readily detected by electron microscopy within 3 to 10 min of a heat shock, though for a reliable detection of these changes with ^{31}P-NMR usually 20 to 30 min are needed. Nevertheless the kinetics of the intensity growth of the corresponding ^{31}P-NMR peak also indicate that these changes begin with the onset of a heat shock. Similarly, the heat shock causes the dramatic loss of cell viability (Fig. 10). But on the other hand the cells began to take up huge macro-

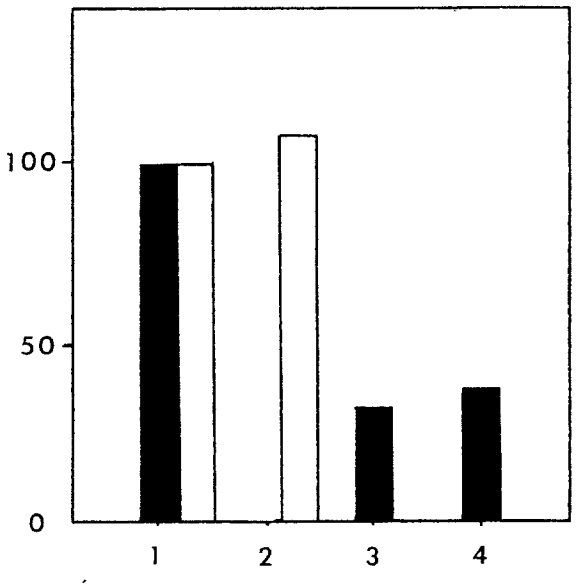


Fig. 11. Effect of phospholipase C on transformation under the conditions promoting the observed polymorphic changes in membranes. Since the cell viability (□) is not affected by the enzyme under the conditions of the experiment, the number of transformants (■) reflects the amount of DNA taken up by the cells. (1) DNA uptake and viability in the absence of the enzyme (100 percent ordinate scale); (2) 6 μg of the enzyme per 5×10^8 cells was added; (3) DNA uptake by the cells washed once with 0.01 M $CaCl_2$; (4) the same in cells washed twice with 0.01 M $CaCl_2$

molecules of exogenous DNA and subsequently form a transformant (Fig. 10, and Cosloy & Oishi, 1973; Sabelnikov & Domaradsky, 1981). The efficiency of uptake (presented as the number of transformants per the total number of viable cells) increases almost linearly with time, reaches maximum at 20 min and then gradually decreases (Fig. 10). Furthermore, DNA uptake occurs only during the heat shock (*see also* Cosloy & Oishi, 1973; Sabelnikov & Domaradsky, 1981) and there is close correlation between the killing effect of the heat shock (in the presence of Ca^{2+}) on the cells and the ability of cells to take up exogenous DNA (Oishi & Irbe, 1977; Sabelnikov, Avdeeva & Ilyashenko, 1977).

There is a correlation between the observed morphologic changes and the induced DNA uptake. For example, Ca^{2+} and Ba^{2+} which are known to induce DNA uptake in *E. coli* readily induce membrane polymorphic changes and the formation of tube-like structures. In contrast Mg^{2+} cations induce neither DNA uptake nor the above changes in *E. coli* D12 P400 and P530 strains (*not shown*). The addition of EDTA to Ca^{2+}-treated cells reduces drastically plasmid transformation (by 3 orders of magnitude) and prevents the formation of tube-like structures. Efficient DNA uptake is achieved only if the cell-DNA mixture is preincubated at 0°C before the heat shock (Cosloy & Oishi, 1973; Taketo, 1974). Similarly the formation of tube-like structures during the heat shock is observed only when the cells were preincubated at 0°C. All these data suggest that morphological changes observed are intimately involved in DNA uptake. This suggestion is compatible with and in fact comprises a ground for the unspecificity of cation-dependent DNA uptake (for references *see* Sabelnikov et al., 1984). It seems that the formation of the transformant by the cell that has undergone membrane polymorphic changes is a play between the probability of sufficient DNA uptake and the probability of the cell death within a certain time interval during the heat shock. It is very likely that the polymorphic changes in bacterial membranes are quite "reversible" if they have not yet reached some critical level, similar to a reversible pore formation during the electroshock (for a review *see* Zimmermann et al., 1984; Neumann, 1984).

The evidence presented above on in vivo membrane contacts formation and membrane fusion indicates that these processes mimic the patterns observed in vitro in model lipid membranes. But the model membranes are composed mostly of pure phospholipids. Bacterial membranes in addition contain large amounts of proteins and lipopolysaccharides. However, it is unlikely that the wild-type lipopolysaccharides with long saccharide chains could be involved in the formation of the observed tube-like structures save for the formation of single tubes frequently observed on ultrathin section micrographs (Fig. 5*A*). In this case ^{31}P-NMR data (Fig. 9) suggest that the observed polymorphic changes are primarily phospholipidic in nature. This is further supported by the results obtained under the same conditions with liposomes made from the total *E. coli* lipid extracts (Sabelnikov, Borovjagin and Tarahovsky, *unpublished results*). Since, however, it is considered (for a review *see* Lugtenberg & Van Alphen, 1983) that the outer leaflet of the outer membrane of some wild-type Enterobacteria (*E. coli, Salmonella typhimureum,* etc.) contains very little, if any, phospholipid, the question as to the nature of the observed polymorphic changes demands special consideration. In view of the results presented above it is reasonable to suggest that the treatment of wild-type cells with high concentration of divalent cations promote the enrichment of the outer leaflet with phospholipids. The validity of this suggestion was verified with the use of the exogenously added phospholipase C. The incubation of wild-type (P400) and ompC,F mutant (P530) cells pretreated with divalent cations at 0°C with phospholipase C results in the appearance of readily detectable amounts of diacylglycerol. Since it is un-

likely that the cell envelope is permeable for the enzyme molecules under the employed assay conditions, 0.01 M $CaCl_2$, and 37°C (Sabelnikov & Domaradsky, 1981; Brass, 1986), the results obtained indicated the actual appearance of phospholipids in the outer leaflet of the outer membrane. Besides if the polymorphic changes observed are really and primarily phospholipidic in nature and are involved in DNA translocation it is reasonable to expect that the binding of the enzyme to the cell surface would affect exogenous DNA uptake. This is indeed the case (Fig. 11).

The addition of the enzyme to Ca^{2+}-treated cells at 0°C (when no hydrolysis of phospholipids is observed) before but not after the addition of exogenous DNA results in a dramatic 10 to 1000 times fall of DNA uptake. The cell viability remains unchanged (Fig. 11). It seems that the enzyme binds to cell reversibly since washing off the enzyme recovers the cell's ability to take up exogenous DNA (Fig. 11). A quantitative evaluation of the maximal cell surface occupied by the enzyme molecules at its inhibiting concentrations (1 μg per 1×10^9 cells) yields the values of about several percent of the total cell surface area.

This excludes the possibility of unspecific shielding of DNA uptake sites by the adsorbed enzyme molecules. Furthermore the estimated value corresponds readily to the surface area occupied by Bayer's zones of adhesion between outer and inner membranes (Bayer, 1979). And these zones seem to be the most likely sites of rapid intermembrane exchange of phospholipids observed in *Salmonella typhimurium*, a close relative of *Escherichia coli*, in the presence of 25 mM $CaCl_2$ (Jones & Osborn, 1977*a,b*).

Thus it is very likely that i) the inhibitory effect of phospholipase C on DNA uptake is due to a specific binding of the enzyme to cell surface sites enriched in phospholipids and that ii) these sites are Bayer's zones of adhesion between outer and inner membranes.

The occurrence of phospholipids in the outer leaflet of the outer membranes of wild-type Enterobacteria cells in certain cases is also suggested by the results of Jones and Osborn (1977*a,b*) on the incorporation of exogenously added phospholipids into wild-type *Salmonella typhimureum* membranes in the presence of 25 mM $CaCl_2$. The appearance of phospholipids in the outer leaflet after the treatment of cells with high concentration of divalent cations is further substantiated by the results of Stan-Lotter and Sandersen (1981). These authors have shown that in the presence of high concentration of uni- and divalent cations outer membrane of *E. coli* and *Salmonella typhimurium* may loose large amounts of proteins, mainly porins as cation-associated outer membrane vesicles. On the other hand, it is well known that the lack of outer membrane proteins is compensated by the increased amounts of phospholipids (Lugtenberg & Van Alphen, 1983).

In conclusion the results of this study not only confirm the occurrence of polymorphic transitions *in situ* membranes of bacteria such as *E. coli* and *P. putida* under certain conditions, but allow to envisage different and consequent stages of this process. They directly support the suggestion of Ahkong et al. (1975) on the identity of mechanisms of membrane contact formation and fusion in model and cellular membranes.

The results strongly suggest that in these processes membrane phospholipids are primarily involved. The evidence presented suggest close relationship between the observed morphological changes and exogenous DNA uptake, occurred under the employed assay conditions.

We think that since polymorphic changes and fusion in model membranes are induced not only by divalent cations but by a variety of other substances and factors (Lucy, 1984; Cullis et al., 1985), these phenomena may occur during the life cycle of a cell at spontaneous and favorable changes of the environment and promote some processes like the genetic exchange between the cells, uptake or excretion of different substances, etc.

We are very grateful to Prof. U. Henning (Tubingen, FRG) for providing us with wild-type and mutant *E. coli* strains and Prof. V. Vasiliev (Institute for Protein Research, Pushino) for providing us with electron-microscopy facilities. The skillful help of Ms. L. Meleshko in drawing Fig. 8 is gratefully acknowledged.

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Received 5 March 1987; revised 19 June 1987