Biochimica et Biophysica Acta, 554 (1979) 68—75

BBA 78393

ALTERATION OF HUMAN ERYTHROCYTE PLASMA MEMBRANES BY PERFRINGOLYSIN O AS REVEALED BY FREEZE-FRACTURE ELECTRON MICROSCOPY

STUDIES ON *CLOSTRIDIUM PERFRINGENS* EXOTOXINS V

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(Received September 11th, 1978)
(Revised manuscript received December 18th, 1978)

Key words: Perfringolysin O; Electron microscopy; θ-Toxin; Erythrocyte membrane; Freeze-fracture; (Clostridium perfringens)

Summary

When human erythrocyte membranes were treated with perfringolysin O (*Clostridium perfringens* θ-toxin) and examined by electron microscopy after freeze-fracture, two ultrastructural alterations were observed in fracture faces of membrane. (1) A random aggregation of intramembranous particles was seen in the fracture face of the protoplasmic half (PF face) of all membranes treated with the toxin, even if at a low concentration (40 hemolytic units/ml). On the other hand, the aggregation in the fracture face of the exoplasmic half (EF face) was observed only in membranes treated with a high concentration (3300 hemolytic units/ml) for 2 h. (2) Round protrusions and 'cavities' with 30 nm in diameter were visible in EF and PF faces of membranes treated with a high concentration, respectively. These structures were always protruded toward cytoplasmic side, but did not appear to form holes through the membrane.

Ring and arc shaped structures with a dark center of 26 nm and a distinct border of 5 nm in width were observed when the toxin alone was negatively stained at a very high concentration (170 000 hemolytic units/ml). These structures were also produced in the presence of cholesterol even if the toxin concentration was low.

Abbreviations: EF face, fracture face of exoplasmic half; PF face, fracture face of protoplasmic half.

Introduction

Perfringolysin O (*Clostridium perfringens* θ-toxin) is one of the oxygen labile cytolysins, which include streptolysin O, pneumolysin, tetanolysin and cereolysin. These toxins have many properties in common incruding their high affinities for cholesterol [1—8]. Purification and characterization of perfringolysin O have been presented in our previous reports [1—3]. It has been proposed that they cause cytolysis by producing similar structural alterations after binding to cholesterol in cell membranes [4]. Negative staining electron microscopy has revealed that they produce ring and arc shaped structures in biological and artificial membranes containing cholesterol, in cholesterol suspensions [9—12], and occasionally also in the absence of membranes or cholesterol [11,13]. Smaller molecular cytolytic compounds with high affinities for sterols such as filipin [14—18] and 'white' saponin [18—20], and lysins with no affinity for sterols such as complement-binding immune lysins [21,22] and streptococcal α-toxin [23—25] produce various similar structures. To investigate structural alterations connected with the hemolytic mechanism, human erythrocyte membranes treated with perfringolysin O under different conditions were observed by freeze-fracture electron microscopy.

Materials and Methods

Preparation of perfringolysin O. Cultivation conditions of *Clostridium perfringens* PB6K N5-L9, purification method of the toxin and assay methods of activities were reported [1,26]. The toxin preparation used in this report had a specific activity of 260 hemolytic units/μg of protein when it was activated by incubation with 20—25 mM cysteine for 10 min at 37° C, and was completely freed from other activities found in the culture filtrate [1,26]. One hemolytic unit is defined as the activity which lyses 50% of sheep erythrocytes contained in 3 ml of 1% (v/v) cell suspension (about $4.5 \cdot 10^9$ cells/vessel) in 70 mM phosphate buffer (pH 6.8) containing 78 mM NaCl after incubation at 37° C for 30 min. Therefore, two hemolytic units of the toxin can lyse all the cells added.

Treatment of erythrocytes or their ghost membranes with perfringolysin O. Human erythrocyte ghost membranes were prepared as described [27]. The ghost membranes prepared from 2 ml of blood ($1 \cdot 10^{10}$ cells, 2.2 mg of protein) were incubated at 37° C for 30 min or 2 h with 3300 and 40 hemolytic units/ml of the toxin in 10 mM phosphate buffered saline (pH 7.4) in 3 ml total volume.

The cells were repeatedly washed with phosphate buffered saline. When 1.5 ml of the cell suspension (about $1.4 \cdot 10^{10}$ cells) was incubated with 6800 hemolytic units of the toxin in 2.5 ml phosphate buffered saline at 37° C, complete hemolysis was observed after 4 min. The reaction mixture was rapidly diluted with 20 vols. of the same buffer and fixed by adding glutaraldehyde to make 1% final concentration.

Freeze-fracturing and negative staining. Aliquots of control and perfringolysin O treated ghost membranes were prefixed with 0.75% (v/v) glutaraldehyde buffered with 0.1 M sodium phosphate (pH 7.4) and washed 3 times with

distilled water. Samples were freeze-fractured with a Hitachi HFZ-1 freeze-etch device [17]. Specimens used for negative staining were prepared as described [17]. Specimens and freeze-fractured replicas were examined with a JEM-100 U electron microscope operating at 80 kV.

Results

Negative staining electron microscopy

Perfringolysin O at very high concentration (170 000 hemolytic units/ml) in the absence of cholesterol showed large numbers of ring and arc shaped structures which consisted of a distinct border of 5 nm in width and a dark center of 22—30 nm (Fig. 1), which is in good agreement with the observation of Cowell et al. [13] on cereolysin. Under this condition most of the toxin molecules may aggregate together to form these structures. The toxin alone at 3300 hemolytic units/ml did not show these structures, but they were observed on the membranes and in membrane free fields (Fig. 2a, b and c) after incubation with erythrocyte ghosts at 37°C for 30 min. The diameter of the dark center in rings varied from 18 to 29 nm, while the width of the distinct border was an almost homogeneous 5.5 nm. These observations are similar to those of Smyth et al. [11] for lesions caused by the toxin in horse erythrocyte ghosts. Lower concentrations of the toxin and shorter incubation times reduced the number of structures observed. No alteration was visible when ghost membranes were treated with 40 hemolytic units/ml or when the cells were completely lysed by treatment with 5 hemolytic units/ml for 30 min at 37°C.

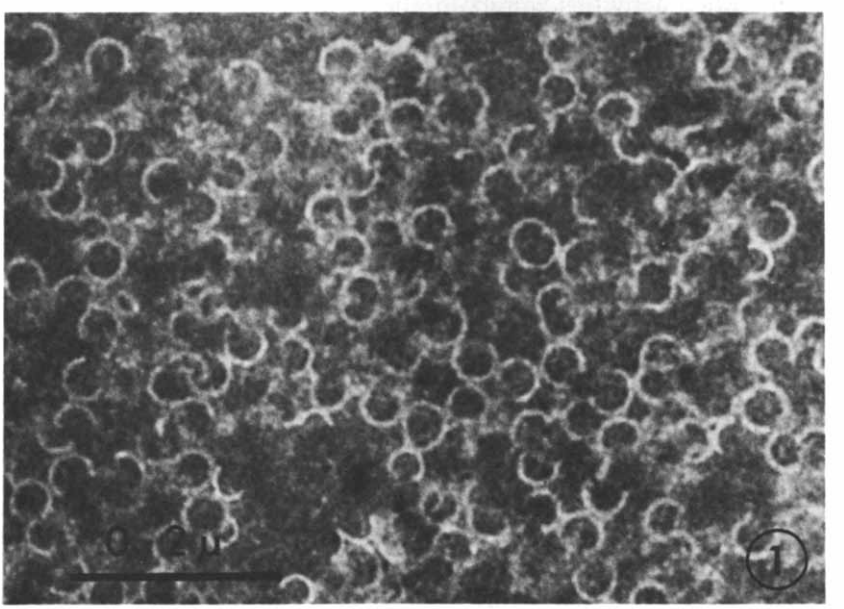


Fig. 1. Negatively stained specimen of 'active' perfringolysin O itself.

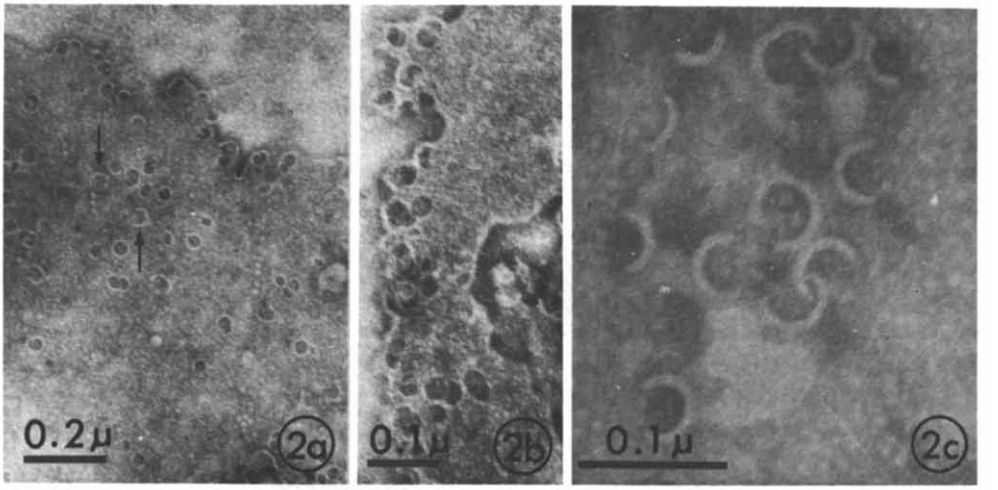


Fig. 2. Negatively stained specimens of human erythrocyte ghost membranes treated with perfringolysin O (3300 hemolytic units/ml) at 37°C for 2 h (a, b and c). The ring structures, their aggregated forms and the arc structures are observed (a). A higher density of these structures is seen at the membrane edge (b). There are also some free arcs (c).

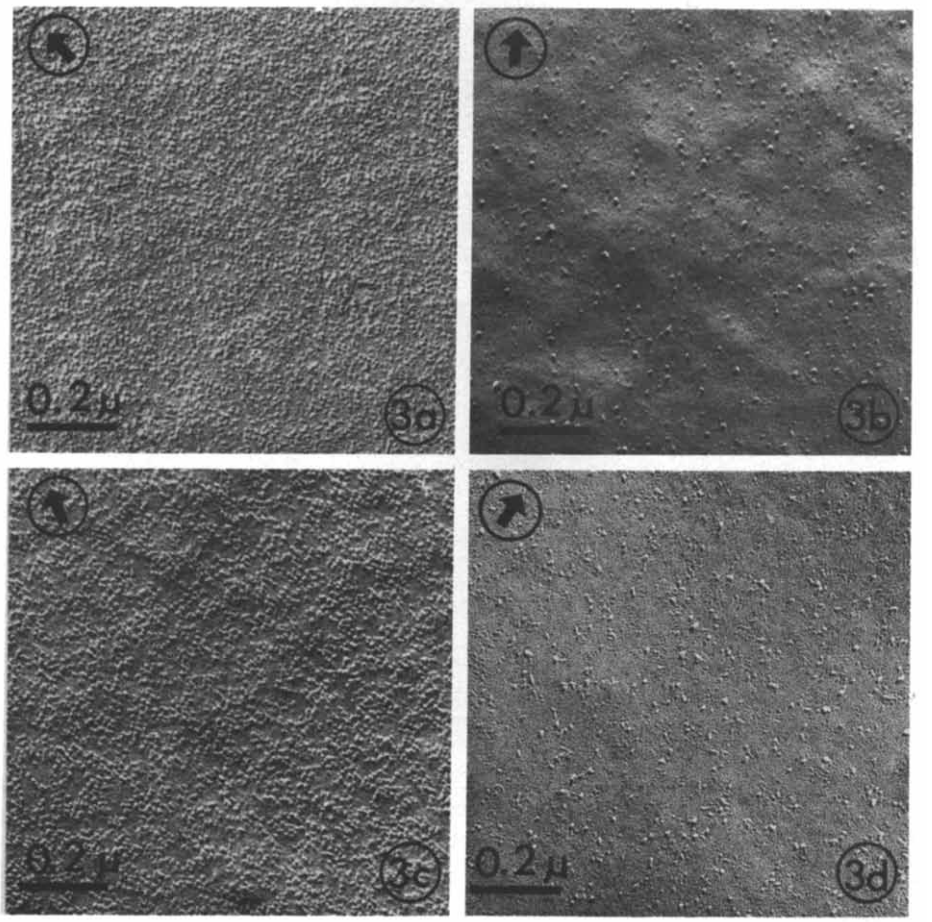
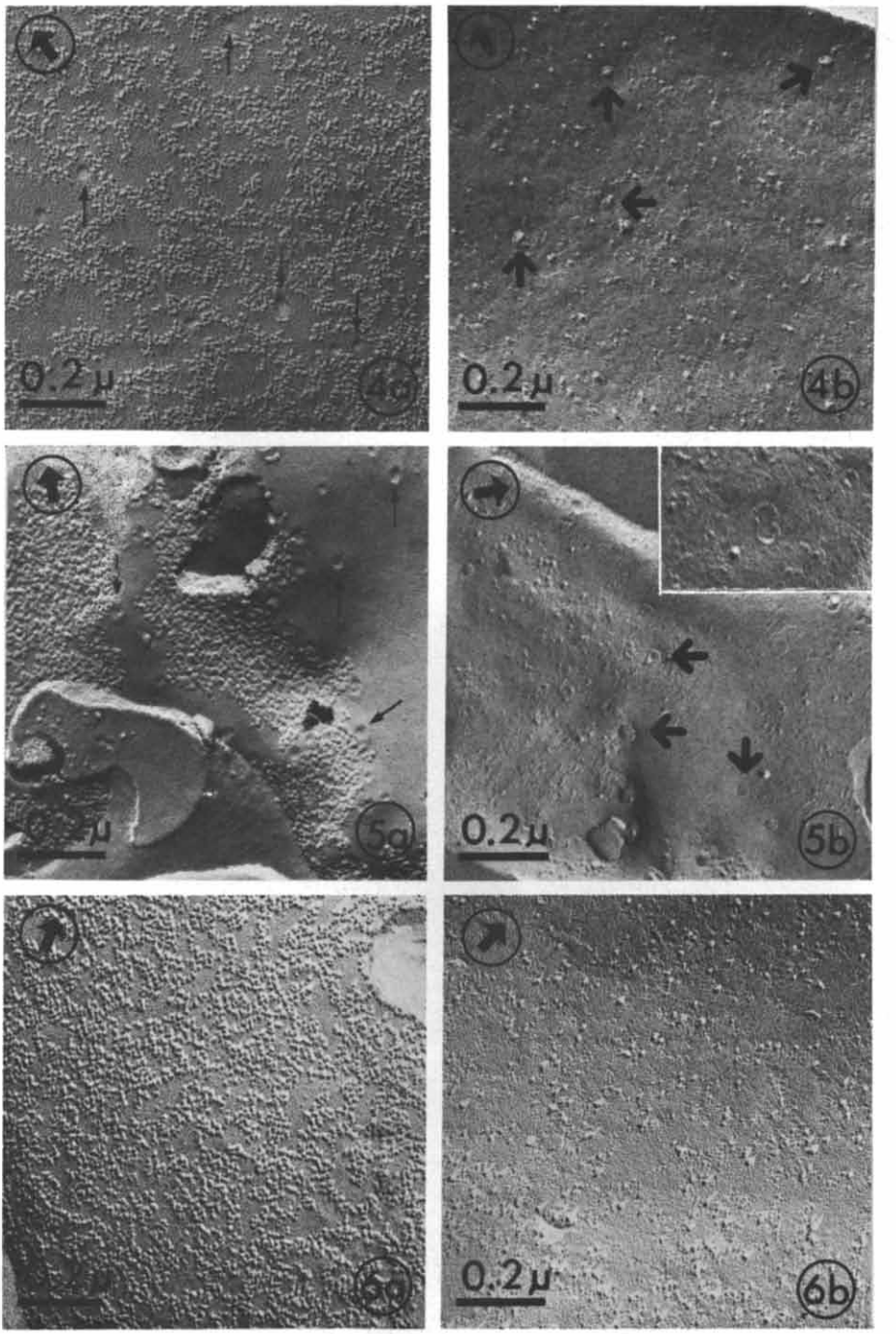


Fig. 3. Freeze-fracture appearances of human erythrocytes (a and b shows PF and EF face, respectively), and of ghost membranes (c, PF face; d, EF face).



Figs. 4—6. Freeze-fracture appearances of human erythrocyte ghost membranes treated with perfringolysin O under different conditions at 37° C. Fig. 4, at 3300 hemolytic units/ml for 30 min; Fig. 5, at 3300 hemolytic units/ml for 2 h; Fig. 6, at 40 hemolytic units/ml for 2 h. In these figures, a and b represents PF and EF face, respectively. In Fig. 4 and 5, round, shallow 'cavities' (thin arrows), and round protrusions (thick arrows) are seen in the PF and EF face, respectively. The inset in Fig. 5b shows a fused arc-shaped structure. Magnification: X100 000.

Freeze-fracture electron microscopy

The PF face of human erythrocytes contains a high density of intramembranous particles with a diameter of 8.5 nm, while the EF face contains similarly sized particles but in much lower density. The particle density on the PF face of intact cell membranes (3510/μm^2, Fig. 3a) was slightly higher than that of ghost membranes (2910/μm^2, Fig. 3c). On the other hand, the particle density on the EF face of intact cell membranes (Fig. 3b) was slightly lower than that of ghost membranes (Fig. 3d).

The toxin induced random aggregation of particles on the PF face, forming particle-free areas. The particle density on the PF face in ghost membranes treated with 3300 hemolytic units/ml for 30 min was reduced to 2300/μm^2 (Fig. 4a). After incubation for 2 h at the same concentration, the PF face segregated into particle-free and aggregate areas (Fig. 5a). Treatment with 40 hemolytic units/ml for 2 h reduced the particle density on the PF face to 2400/μm^2 (Fig. 6a). In completely lysed cells after treatment with 1700 hemolytic units/ml for 4 min at 37° C, the particle density on the PF face was also reduced (Fig. 7a, 2530/μm^2), while on the EF face (Fig. 7b) was the same as for the control (Fig. 3b). Distribution of particles on the EF face was altered only in a specimen of ghost membranes treated with the toxin at 3300 hemolytic units/ml for 2 h (Fig. 5a). The particle-free areas visible in this specimen were irregularly shaped.

Another membrane alteration was formation of round structures which were seen on both faces of ghost membranes treated with the toxin at 3300 hemolytic units/ml for 30 min or 2 h at 37° C. On the PF face (Fig. 4a or 5a, respectively) round shallow 'cavities' was randomly distributed, and on the EF face (Fig. 4b or 5b, respectively) round protrusions were visible. These protrusions were heterogeneous rings with 30 nm in diameter, and consisted of a slight rim and a slightly convexed upperside with 20 nm in diameter on the

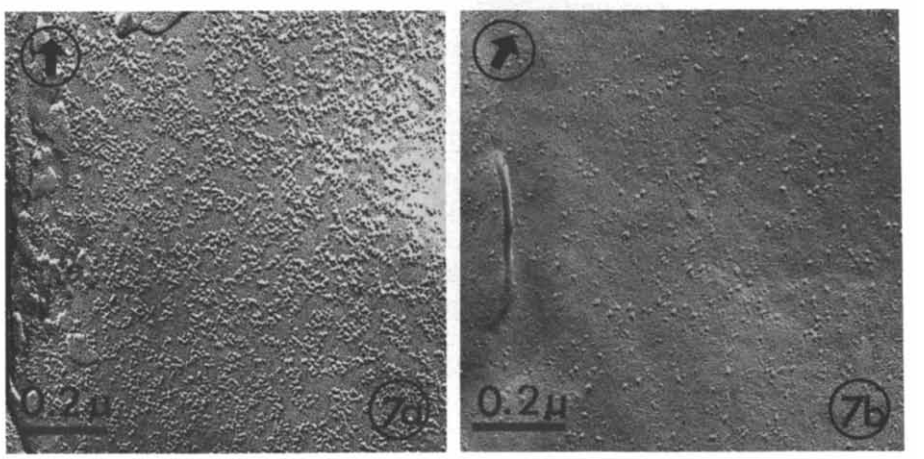


Fig. 7. Freeze-fracture appearances of completely lysed erythrocytes after treatment with perfringolysin O (1700 hemolytic units/ml) at 37° C for 4 min. On comparison with the control (Fig. 3a), the visible alteration is only decrease of the density of the particles in the PF face (a). The EF face (b) is the same as the control (Fig. 3b).

average. Protrusions and the 'cavities' revealed the same structures produced in membranes, which protrude to the protoplasmic side to make the 'cavities' on the cell surface. Some fused forms composed of two protrusions were seen (Fig. 5b). The 'cavities' could not be observed in completely lysed cells after treatment with 1700 hemolytic units/ml for 4 min at 37° C (Fig. 7), suggesting that formation of the 'cavities' occurs only during a period of incubation for or longer than 30 min. The shape of the 'cavities' became slightly more characteristic after the longer incubation (compare Fig. 5 to Fig. 4), but their number was not changed. Membranes treated with the toxin at 40 hemolytic units/ml for 2 h showed no 'cavities' (Fig. 6), either because they were not formed at lower concentrations or because there were too few to be found. No transverse holes were visible in the 'cavities' on either face.

Discussion

Results in this report show that the 'cavities' in freeze-fractured specimens of human erythrocyte ghosts treated by perfringolysin O seem to be 'altered bilayer portions' in the membrane structure. The 'cavities' and the rings visible in negatively stained specimens are similar in size. However, number of the 'cavities' is remarkably smaller than that of the rings, and their distribution differs. Moreover, any structure corresponding to the arcs is invisible in all of the freeze-fractured specimens. Therefore, it is difficult to be certain of the correspondence of the 'cavities' to the rings in lesions induced by the toxin.

The aggregation of intramembranous particles in the PF face may be the first alteration induced by the toxin, because it is visible in all of the specimens prepared. In contrast, the exoplasmic half seem to be resistant to the toxin because the particle aggregation was observed only in membranes treated at a high concentration for a long period (Fig. 5b).

The amount of the toxin necessary to induce complete hemolysis is calculated to be seven molecules per cell under the assay condition, assuming a toxin molecular weight of 53 000 and a specific activity of 889 000 hemolytic units/mg of protein [1]. The toxin was completely adsorbed to the cells within 10 min, while hemolysis was induced after a few min of lag time and required a period of 30 min for completion at 37° C. Adsorption was practically complete while hemolysis was hardly observed at 0° C for 24 h [2]. These results suggest that seven or more molecules of the toxin bind to cholesterol situated at a few sites on the exoplasmic half of the cell requiring little energy. This binding induces a membrane structural alteration to make the first leak requiring remarkable energy. Once this leakage occurs, large amounts of the toxin bind to cholesterol in the protoplasmic half, inducing aggregation of particles in the PF face, and then gradually producing the rings if the toxin concentration is high. After hemolysis, more toxin may bind to cholesterol in the exoplasmic half repeling many preventing groups on the cell surface during a long period of incubation, and gradually producing the 'cavities' which would be fixed into the membrane structure. The ring formation of the toxin alone will be clarified in the near future.

References

1 Mitsui, K., Mitsui, N. and Hase, J. (1973) Japan. J. Exp. Med. 43, 377—391
2 Hase, J., Mitshui, K. and Shonaka, E. (1975) Japan. J. Exp. Med. 45, 433—438
3 Hase, J., Mitsui, K. and Shonaka, E. (1976) Japan. J. Exp. Med. 46, 45—50
4 Bernheimer, A.W. (1974) Biochim. Biophys. Acta 344, 27—50
5 Smyth, C.J. (1975) J. Gen. Microbiol. 87, 219—238
6 Cowell, J.L. and Bernheimer, A.W. (1977) Infect. Immun. 16, 397—399
7 Bernheimer, A.W. (1970) in Bacterial Toxins (Ajl, S.J., Kadis, S. and Montie, T.C., eds.), Vol. I, pp. 183—209, Academic Press, New York and London
8 Ispolatovskaya, M.V. (1971) in Microbial Toxins (Kadis, S., Montie, T.C. and Ajl, S.J., eds.), Vol. IIA, pp. 128—129, Academic Press, New York and London
9 Dourmashkin, R.R. and Rosse, W.F. (1966) Am. J. Med. 41, 699—710
10 Duncan, J.L. and Schlegel, R. (1975) J. Cell. Biol. 67, 160—173
11 Smyth, C.J., Freer, J.H. and Arbuthnott, J.P. (1975) Biochim. Biophys. Acta 382, 479—493
12 Pendleton, I.R., Kim, K.S. and Bernheimer, A.W. (1972) J. Bacteriol. 110, 722—730
13 Cowell, J.L., Kim, K.S. and Bernheimer, A.W. (1978) Biochim. Biophys. Acta 507, 230—241
14 Kinsky, S.C., Luse, S.A., Zopf, D., van Deenen, L.L.M. and Haxby, J. (1967) Biochim. Biophys. Acta 135, 844—861
15 Bittman, R., Chen, W.C. and Anderson, O.R. (1974) Biochemistry 13, 1364—1373
16 Tillack, T.W. and Kinsky, S.C. (1973) Biochim. Biophys. Acta 323, 43—54
17 Kitajima, Y., Sekiya, T. and Nozawa, Y. (1976) Biochim. Biophys. Acta 445, 452—465
18 Seeman, P. (1974) Fed. Proc. 33, 2116—2124
19 Bangham, A.D. and Horne, R.W. (1962) Nature 196, 952—953
20 Glauert, A.M., Dingle, J.T. and Lucy, J.A. (1962) Nature 196, 953—955
21 Rosse, W.F., Dourmashkin, R. and Humphrey, J.H. (1966) J. Exptl. Med. 123, 969—984
22 Iles, G.H., Naylor, S.D. and Cinader, B. (1973) J. Cell. Biol. 56, 528—539
23 Freer, J.H., Arbuthnott, J.P. and Bernheimer, A.W. (1968) J. Bacteriol. 95, 1153—1168
24 Arbuthnott, J.P., Freer, J.H. and Billcliffe, B. (1973) J. Gen. Microbiol. 75, 309—319
25 Freer, J.H., Arbuthnott, J.P. and Billcliffe, B. (1973) J. Gen. Microbiol. 75, 321—332
26 Mitsui, K., Mitsui, N. and Hase, J. (1973) Japan. J. Exp. Med. 43, 65—80
27 Dodge, J.P., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130