Biochimica et Biophysica Acta, 382 (1975) 479–493

BBA 76910

INTERACTION OF *CLOSTRIDIUM PERFRINGENS* θ-HAEMOLYSIN, A CONTAMINANT OF COMMERCIAL PHOSPHOLIPASE C, WITH ERYTHROCYTE GHOST MEMBRANES AND LIPID DISPERSIONS

A MORPHOLOGICAL STUDY

CYRIL J. SMYTH[a,*], JOHN H. FREER[b,**] and JOHN P. ARBUTHNOTT[c]

[a]*Department of Bacteriology, Statens Bakteriologiska Laboratorium, S-105 21, Stockholm (Sweden),* [b]*Department of Microbiology, University of Glasgow, Alexander Stone Building, Garscube Estate, Bearsden, Glasgow G61 1QH and* [c]*Department of Bacteriology, University of Glasgow, The Royal Infirmary, Glasgow, G4 OSF (U.K.)*

(Received July 25th, 1974)
(Revised manuscript received December 12th, 1974)

SUMMARY

Commercially available preparations of phospholipase C from *Clostridium perfringens* are commonly contaminated with θ-haemolysin, one of a group of bacterial haemolysins called oxygen labile (0-labile) haemolysins. Treatment of erythrocyte ghosts and a mixed lipid dispersion containing cholesterol with commercially available phospholipase C in the absence of Ca^{2+} and the presence of phosphate buffer and/or EDTA resulted in the formation and release of ring or arc-shaped structures. Highly purified phospholipase C, free of θ-haemolysin, produced no changes in the morphology of erythrocyte ghosts or lipid dispersions in the presence of phosphate or EDTA, but caused the formation of typical diglyceride droplets in the presence of Ca^{2+} in the absence of these inhibitors. Ring structures, identical to those caused by commercial phospholipase C, were formed on addition of highly purified θ-haemolysin to erythrocyte ghost membranes, lipid dispersions containing cholesterol and cholesterol dispersions, but not on treatment of membranes from *Micrococcus lysodeikticus*. Heat-inactivated θ-haemolysin (60 °C for 10 min) produced no such effects. The dimensions of rings and arcs displayed heterogeneity. The outside diameters in various preparations varied from approx. 27–58 nm with border thickness of 4.1–7.8 nm.

INTRODUCTION

Phospholipase C (EC 3.1.4.3), the α-toxin of *Clostridium perfringens* type A, has been used as a probe of membrane structure and function in many fields of

* Present address: Department of Microbiology, New York University Medical Center, 550 1st Avenue, New York, N.Y. 10016 U.S.A.
** To whom reprint requests should be addressed.

biology [1]. As such its value depends on the specificity of the changes produced which are related to the purity of the preparations used [2]. It has been frequently used to disrupt membrane bounded viruses to allow the study of internal components. Characteristic features described after such treatment have been open and closed ring or arc-shaped structures, sometimes seen as continuous rings, arcs, coils or fragments thereof [3–9]. It was at first suggested that these structures were released from the viral nucleoid [3] and that they could be ribonucleoprotein elements of the nucleocapsid [4].

However, their association with membrane fragments of chick embryo fibroblasts [5] and uninoculated control cell monolayers treated with commercial phospholipase C led to the conclusion that these structures were probably derived from degradation of the viral envelopes acquired from host cells on maturation. Indeed, their identification on and release from phospholipase C-treated erythrocyte ghosts [7, 10, 11] and rat liver plasma membranes [12, 13] supported this view.

Lipid components common to both membrane-bounded virions and host cells appeared to be involved in the formation of these ring structures. Reconstruction experiments with various mixtures of phospholipids and cholesterol corresponding to the major lipid components of the membrane of influenza A virus established that cholesterol was essential in this interaction [6], a conclusion supported by Kemp and Howatson [7]. An unidentified heat-labile component in commercial phospholipase C preparations was shown to be responsible for the interaction with cholesterol leading to the formation of these configurations [6].

These observations prompted us to investigate the possible role of *C. perfringens* θ-haemolysin in the formation of these structures. This haemolysin is one of a group of bacterial haemolysins called oxygen-labile (O-labile) haemolysins, which, although produced by a range of Gram-positive bacterial species, share common biological properties [14, 15]. Three such properties suggested the involvement of θ-haemolysin in the above observations: (a) heat lability, (b) irreversible inactivation by cholesterol and (c) interaction only with membranes containing cholesterol [14–17]. Moreover, the purity of commercially available phospholipases C from *C. perfringens* has recently been examined in detail [2]. Preparations from different sources were shown to contain in addition to phospholipase C, at least seven other enzymes and θ-haemolysin. Thus it seemed highly probable that preparations used previously were also contaminated with θ-haemolysin.

The purification and separation of *C. perfringens* phospholipase C and θ-haemolysin using the technique of isoelectric focusing, made reagents of highly defined purity available for the present studies [18, 19].

MATERIALS AND METHODS

Materials

Ovolecithin was purified from fresh hens eggs by the method of Arbuthnott et al. [20]. Vegetable lecithin was obtained from Schwarz-Mann, Orangeburg, New York; sphingomyelin (from bovine brain) and β-γ-dipalmitoyl DL-α lecithin (synthetic) from Sigma Chemical Company, St. Louis, Mo.; phosphatidylethanolamine from Koch Light Laboratories Ltd., Colnbrook, England; cholesterol from BDH, Poole, England. Crude *C. perfringens* phospholipase C was obtained from Sigma

Chemical Company (lot no 61-C-6900). On assay it contained 3.8 units/mg. Horse blood was obtained in Alsever's solution from Oxoid Ltd., London, England; rabbit, human and sheep blood were obtained by venipuncture on the day of use. Egg yolk substrate was prepared as described by Möllby and Wadström [21]. Chloroform was redistilled before use. All chemicals used were of analytical grade.

Purification of phospholipase C and θ-haemolysin

Phospholipase C was purified from culture supernatant fluids of *C. perfringens* type A strain BP6K by the method of Smyth and Arbuthnott [19]. Only enzyme from single peak fractions of activity was used (component α_A, p*I* 5.5); 40 μg of enzymic protein showed no detectable hyaluronate lyase, collagenase, neuraminidase, or θ-haemolysin activities. The enzyme contained 11.7 units/ml, spec. act. of 13.8 units/mg protein. θ-Haemolysin was purified from culture supernatants fluids of type A strain ATCC 13124 [18]. Material from refocused fractions containing peak activity of component θ_1 (p*I* 6.8–6.9) was used. 50 μg of θ-haemolysin showed no collagenase, DNAase, RNAase, hyaluronate lyase, amylase, esterase, endo-β-*N*-acetyl glucosaminidase, neuraminidase, phospholipase C, lipase, or caseinolytic activity [18]. Both the phospholipase C and the θ-haemolysin preparations were highly purified as gauged by sodium dodecylsulphate disc gel electrophoresis, flat-bed gel isoelectric focusing, immunoelectrophoresis and crossed immunoelectrophoresis [18, 19].

Assay of phospholipase C and θ-haemolysin

Phospholipase C was assayed on egg yolk substrate by the titrimetric assay of Möllby and Wadström [21]. θ-Haemolysin was activated and titrated in two-fold doubling dilutions as described by Smith and Arbuthnott [19], except that β-mercaptoethanol was used as the activator at a concentration of 1 mM. One unit of phospholipase C was defined as the amount of enzyme liberating 1 μmol of titrable H^+/min. One haemolytic unit of θ-haemolysin was defined as the dilution of haemolysin causing haemolysis of 50 % of the erythrocytes in the test in 30 min at 37 °C.

Preparation of erythrocyte ghost membranes

Erythrocyte ghosts were prepared by osmotic lysis according to a modification [22] of the method of Hoogeveen et al. [23]. Light buff-coloured membrane pellets obtained from lysates in 10 mM Sørensen's phosphate buffer, pH 7.4 were resuspended and washed twice in either 200 vol. of 10 mM Tris · HCl buffer, pH 7.4 or 10 mM Sørensen's phosphate buffer, pH 7.4. Erythrocyte ghosts prepared in this manner were haemoglobin-free as assessed by polyacrylamide gel electrophoresis [22] and were used within 2–3 h of preparation.

Preparation of lipid dispersions

A lipid dispersion comprising the major lipid components of influenza A virus [6], but lacking lysolecithin, consisted of cholesterol : sphingomyelin : lecithin : phosphatidylethanolamine in the molar proportions 4.8 : 2.1 : 1.3 : 1.0, assuming dipalmitoyl fatty acid residues on each phospholipid. The lipids were dissolved individually in redistilled chloroform, mixed and dried in vacuo to a film in a round-bottomed flask. The lipid mixture was resuspended in 2.0 ml distilled water to give a final lipid concentration of 5 mg/ml (4.6 μmol lipid/ml) and dispersed by sonicating for 90 s in

an ultrasonic bath (Sonicor Instrument Corporation, Glen Head, N.Y., U.S.A.). The dispersion was allowed to equilibrate at room temperature for approx. 1 h. Dispersions of lecithin, 10 mg/ml, were similarly prepared in distilled water. Cholesterol (3 mg/ml) was dispersed by adding an acetone solution of the lipid to distilled water at 95 °C [20].

Preparation of bacterial membranes

Total membrane fractions were prepared from *Micrococcus lysodeikticus* (NCTC 2665) by the method of Salton and Freer [24]. Washed membranes were resuspended in Tris · HCl buffer, 50 mM, pH 7.4 to a final concentration of 2–3 mg dry wt membrane/ml. This suspension was used on the day of preparation.

Reaction mixtures

Reaction mixtures comprised 0.1 ml lipid dispersion or 0.1 ml erythrocyte ghosts and 0.1 ml crude or purified phospholipase C or θ-haemolysin in 10 mM Tris · HCl buffer, pH 7.4, or 10 mM Sørensen's phosphate buffer, pH 7.4. The θ-haemolysin was activated just prior to use by adding 10 μl of 10 mM β-mercaptoethanol to 0.1 ml θ-haemolysin and incubating for 5 min at 37 °C. The activated toxin was allowed to cool to room temperature before adding the lipid dispersion or erythrocyte ghosts. When necessary reaction mixtures contained 2 mM $CaCl_2$ or 1 mM EDTA. After removing zero time samples, reaction mixtures were incubated at 37 °C in a water-bath.

Negative staining and electron microscopy

Samples were negatively stained by a modification of the method of Brenner and Horne [25] by diluting with 2 % (w/v) ammonium molybdate. Drops were applied to grids covered with carbon-coated formvar. Sufficient suspension was withdrawn with filter paper points to leave a thin film on the grids which dried within 5 s. Grids were examined immediately after preparation in a Phillips EM 300 electron microscope operating at 60 kV.

Protein determinations

The method of Lowry et al. [26] was used with bovine serum albumin (Sigma Chemical Company, grade 1) as standard.

Thin layer chromatography

Thin layer chromatography was done on activated MN-Polygram sil N-HR sheets (Camlab Ltd., Cambridge, England) using as solvent chloroform/methanol (95 : 5, by vol). Cholesterol was identified by comparison with authentic standard after spraying with 50 % v/v H_2SO_4 and heating at 110 °C for 5 min.

RESULTS

Control erythrocyte ghosts

Control ghosts prepared from horse, human, rabbit and sheep erythrocytes possessed common morphological features. They all had a fine granular appearance (Fig. 1a) although the sheep erythrocyte ghosts appeared more fragmented with many

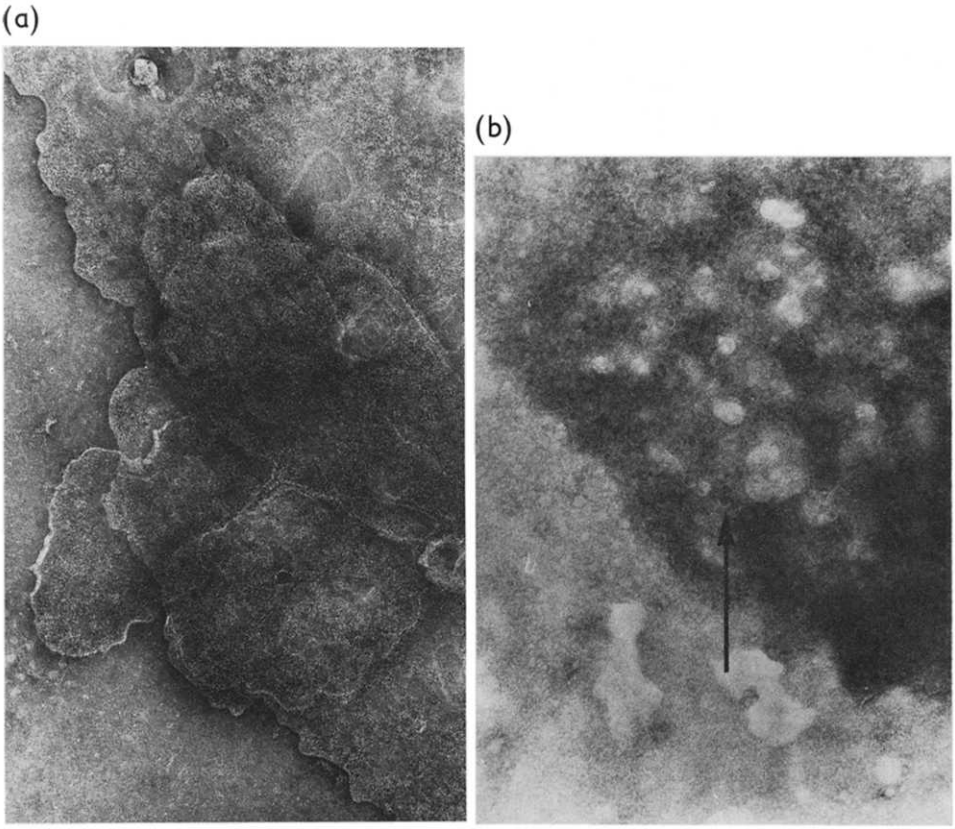


Fig. 1. Horse erythrocyte ghosts, untreated and treated with commercial phospholipase C. (a). Tris-washed ghosts incubated at 37 °C for 5 min with 0.5 mM β-mercaptoethanol, magnification × 65 000. (b). Tris-washed ghosts treated with commercial phospholipase C at a final concentration of 2.5 mg/ml after incubation at 37 °C for 5 min. Area showing the development of groups of rings or pits is arrowed, magnification × 70 000.

stromolytic features. No differences were noted between ghosts washed with Tris · HCl or Sørensen's phosphate buffers. Neither did the addition of 0.5 mM β-mercaptoethanol, 2 mM $CaCl_2$ or 1 mM EDTA nor combinations of these reagents affect the overall appearance of ghosts on electron microscopy.

Treatment of erythrocyte ghosts with commercial and highly purified phospholipase C

Phosphate-washed erythrocyte ghosts were treated with commercial phospholipase C at two final concentrations in reaction mixtures, 0.5 mg/ml and 2.5 mg/ml, i.e. under conditions employed by authors who previously described the formation of ring structures [3–9]. Negatively stained preparations thus treated contained ring-like, or arc-shaped structures in the plane of the membranes or being released from ghosts (Fig. 1b). These had external diameters of approx. 35 nm and distinct borders approx. 5.7 nm wide. The effect was more pronounced at the higher concentration of commercial phospholipase C especially after incubation at 37 °C. Identical findings were obtained with Tris-washed ghosts in the presence or absence of 1 mM EDTA. On titration for θ-haemolysin, the commercial preparation of phospholipase C used

in these studies contained 4000 haemolytic units/mg (unactivated titre).

By contrast, phosphate-washed ghosts and Tris-washed ghosts treated with highly purified phospholipase C appeared no different from control ghosts. However, negatively stained preparations of Tris-washed ghosts treated with purified enzyme in the presence of 2 mM $CaCl_2$ contained electron transparent droplets, which either remained associated with the ghosts or were released as free droplets. Little fragmentation of human, sheep, horse or rabbit erythrocyte ghosts occurred. On no occasion were arc or ring structures similar to those seen on treatment with commercial phospholipase C demonstrated on ghosts treated with highly purified phospholipase C.

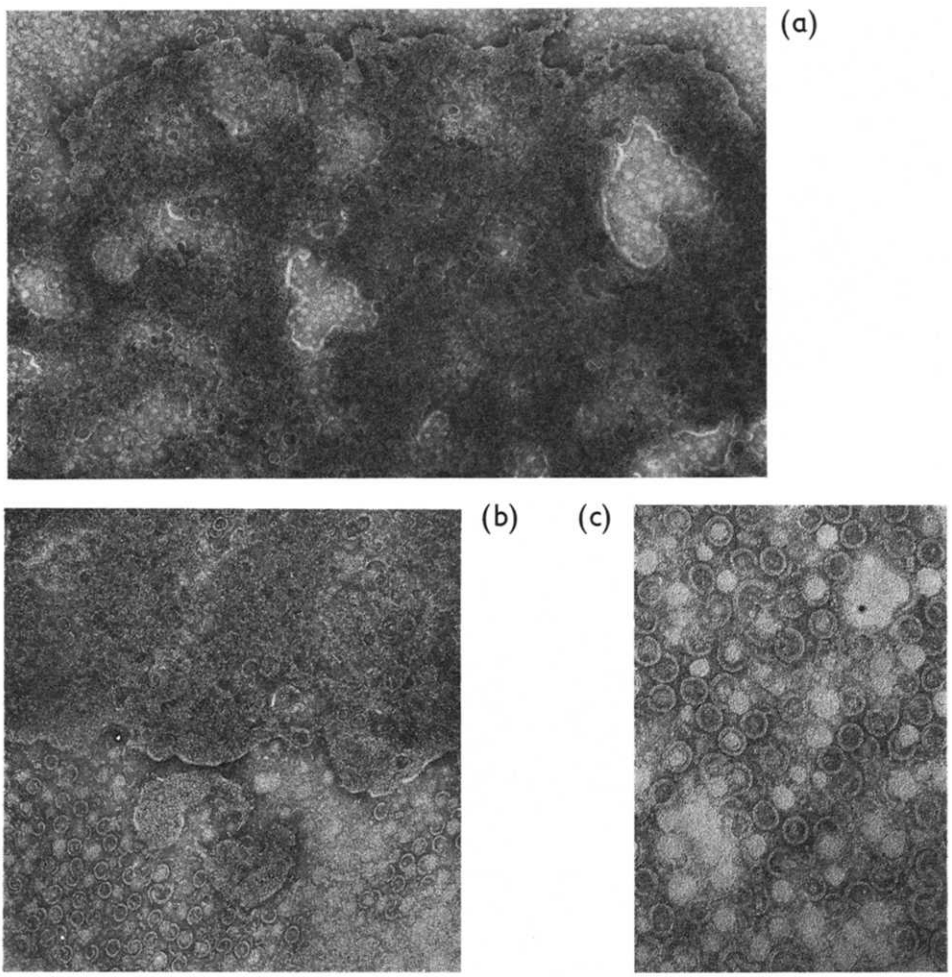


Fig. 2. Horse erythrocyte ghosts treated with highly purified θ-haemolysin. Ghosts treated at 50 000 haemolytic units/ml final concentration and incubated at 37 °C for 30 min. (a). Tris-washed ghosts treated in the presence of 0.5 mM β-mercaptoethanol and 2 mM $CaCl_2$, magnification × 65 000. (b). Phosphate-washed ghosts treated in the presence of 0.05 mM β-mercaptoethanol, magnification × 87 000. (c). Ring and arc shaped structures released from Tris-washed ghosts when treated as under (a), magnification ×176 000. Outside diameters of the rings vary from approx. 27–35 nm; ring thickness varies between 4.5–6.2 nm.

Treatment of erythrocyte ghosts with highly purified θ-haemolysin

Negatively stained ghost preparations treated with activated highly purified θ-haemolysin showed ring or arc-shaped structures, identical to those seen on treatment with crude phospholipase C, on or at the edges of ghosts. At low concentrations of θ-haemolysin (1000–2000 haemolytic units/ml, 2–4 μg/ml final concentration) only "holes" or "pits" with a characteristic ring border were apparent. At higher concentrations (25 000–50 000 haemolytic units/ml, 50–100 μg/ml final concentration) ghosts were frequently fragmented, identical structures were visible in the plane of the membrane (Fig. 2a), arc and ring-shaped structures were seen peeling off at the edges of ghosts (Fig. 2b) and free rings and arcs were readily visible in many fields (Fig. 2c). That formation of these structures occurred rapidly at room temperature (25 °C)

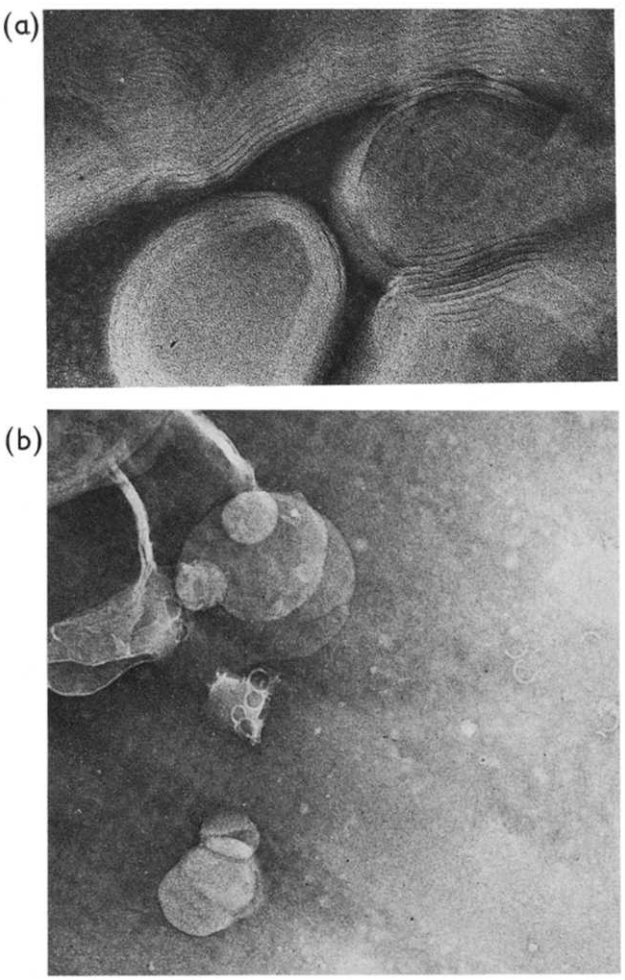
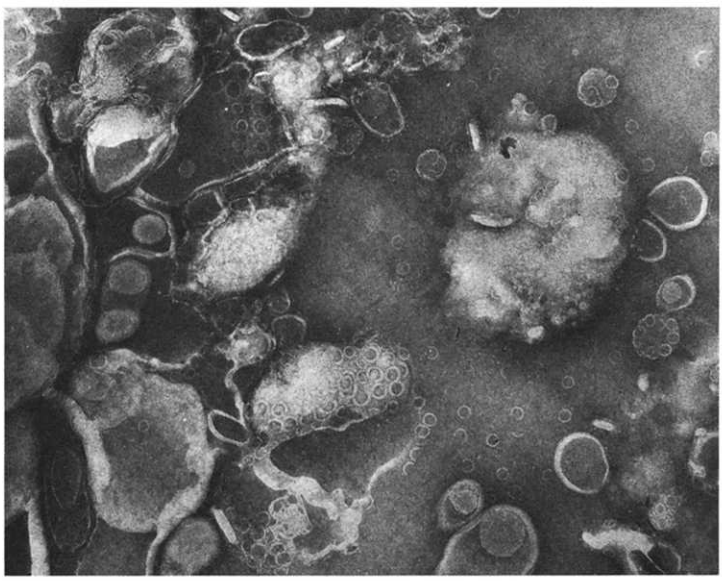


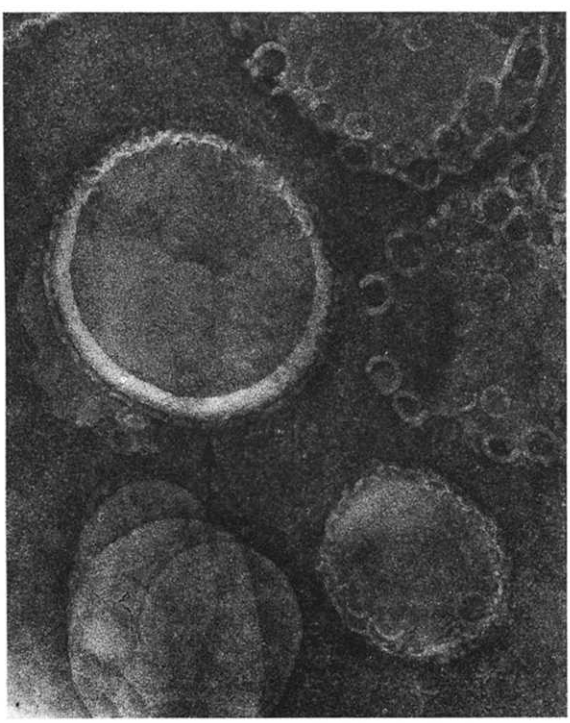
Fig. 3. Negatively stained preparations of mixed lipid dispersion (containing cholesterol, sphingomyelin, lecithin and phosphatidylethanolamine in molar ratios 4.8 : 2.1 : 1.3 : 1.0). (a) Untreated dispersion in Tris buffer with 1 mM EDTA and 0.5 mM β-mercaptoethanol, magnification ×208 000. (b) Dispersion treated with 1 mg/ml final concentration commercial phospholipase C in Tris buffer, magnification × 82 000.

was indicated by the fact that they were found in large numbers in zero time samples. Increased fragmentation and the formation of complex networks of rings and coils was observed when reaction mixtures were incubated at 37 °C. Addition of 1 mM EDTA and/or 2 mM $CaCl_2$ did not affect this interaction. θ-haemolysin produced identical results on both Tris-washed and phosphate-washed ghosts (Figs 2a and 2b).

(a)



(b)



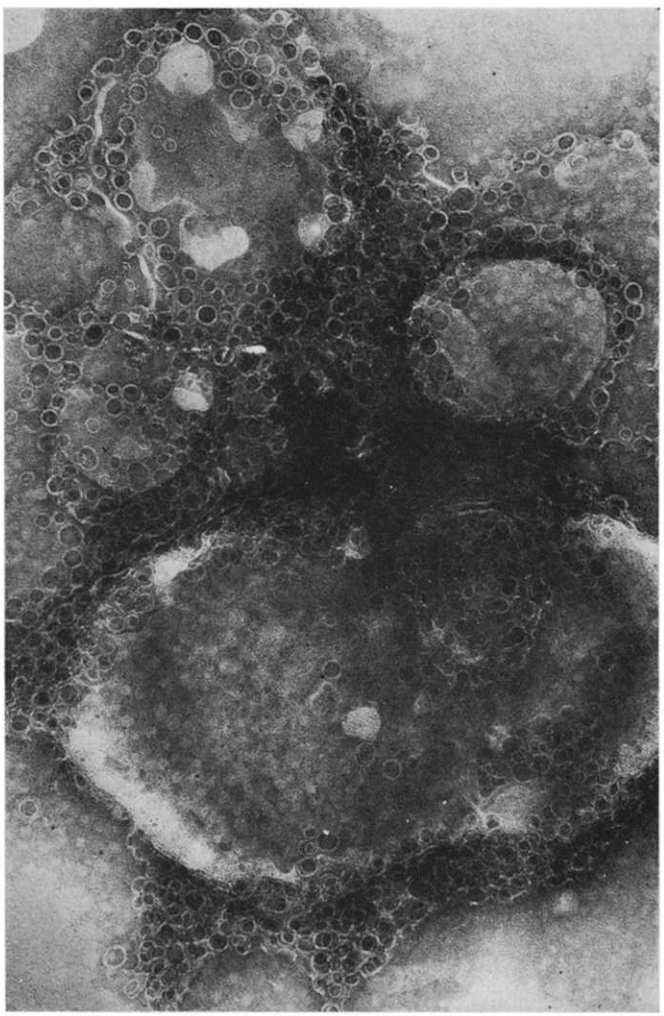


Fig. 4. Mixed lipid dispersion treated with highly purified θ-haemolysin. (a). Dispersion treated at 50 000 haemolytic units/ml final concentration, 2 mM $CaCl_2$, and 0.5 mM β-mercaptoethanol, zero time control i.e. sample removed immediately after addition of θ-haemolysin at 25 °C, magnification × 73 000. Two sizes of rings are apparent, 41 nm outside diameter/6.8 nm thickness and 33 nm outside diameter/5.5 nm thickness, approximately. (b) Dispersion treated in the presence of 1 mM EDTA and 0.5 mM β-mercaptoethanol. Note fragmentation of the lamellae and fragments (arrowed) which appear to be rounding off at the edges, magnification × 189 000. Rings 30–37 nm outside diameter, 6.9 nm thickness. (c) θ-haemolysin treated dispersion in 1 mM EDTA and 0.5 mM β-mercaptoethanol after incubation at 37 °C for 5 min and standing at 25 °C for 20 min. Note the masses of entangled arcs and rings, magnification × 90 000. Rings 29–58 nm outside diameter, 6.7–7.8 nm thickness.

Treatment of mixed lipid dispersions with commercial phospholipase C, purified phospholipase C and θ-haemolysin

Control lipid dispersions comprising ovolecithin, sphingomyelin and cholesterol with or without phosphatidylethanolamine showed typical lamellar arrangements

(Fig. 3a). The addition of 1 mM β-mercaptoethanol, 2 mM $CaCl_2$ or 1 mM EDTA did not affect their morphology. Addition of commercial phospholipase C in the presence of phosphate buffer or Tris buffer+EDTA resulted in the formation of ring and arc-shaped structures (Fig. 3b), whereas highly purified phospholipase C produced no such effects under these conditions.

Mixed lipid dispersions treated with activated highly purified θ-haemolysin possessed numerous arc or ring-like structures (Fig. 4a), even at zero time. Free arcs or rings were frequently seen in the outer layers of concentric lipid lamellae (Fig. 4b). This effect was even more marked after incubation at 37 °C for 5 min. Complex aggregates of complete and incomplete intertwined rings or dense accumulations or rings apparently embedded in the matrix of the dispersions were common features, especially after incubation at 37 °C (Fig. 4c).

A sample of θ-haemolysin was heated at 60 °C for 10 min. The titre fell from 50 000 heamolytic units/ml to < 10 haemolytic units/ml. Mixed lipid dispersions treated with this heat-inactivated θ-haemolysin appeared no different from control dispersions and no ring forms were detected.

Treatment of lecithin or cholesterol (single lipid) dispersions with highly purified phospholipase C and θ-haemolysin

After treatment of the cholesterol dispersions with highly purified θ-haemolysin the semi-crystalline cholesterol droplets showed dense accumulations of ring and arc-shaped structures (Figs 5a and 5b). These remained closely associated and free rings or arcs were uncommon. Again the effect was specific for θ-haemolysin; highly purified phospholipase C caused no ring formation.

When highly purified phospholipase C was added to lecithin dispersions in the presence of Ca^{2+}, lipid droplets were formed, but this effect was not observed in the absence of Ca^{2+} or in the presence of EDTA. When highly purified θ-haemolysin was added to ovolecithin dispersions, which were contaminated with cholesterol to the extent of less than 1 % w/w (as estimated by thin-layer chromatography), arc shaped structures, often occurring in ribbons, were readily seen in the background stain

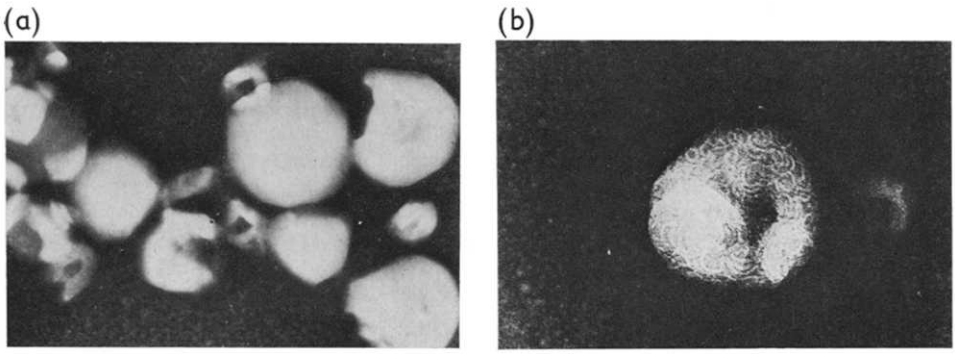


Fig. 5. Cholesterol dispersion treated with highly purified θ-haemolysin. (a) Control dispersion incubated at 37 °C for 5 min in Tris buffer in the presence of 0.5 mM β-mercaptoethanol. Semi-crystalline structures are readily visible, magnification × 154 000. (b) Dispersion treated with θ-haemolysin after incubation at 37 °C for 5 min in Tris buffer in the presence of 0.5 mM β-mercaptoethanol. Note the conversion of the semicrystalline cholesterol droplets into compact arrays of entangled arcs and rings, magnification × 154 000.

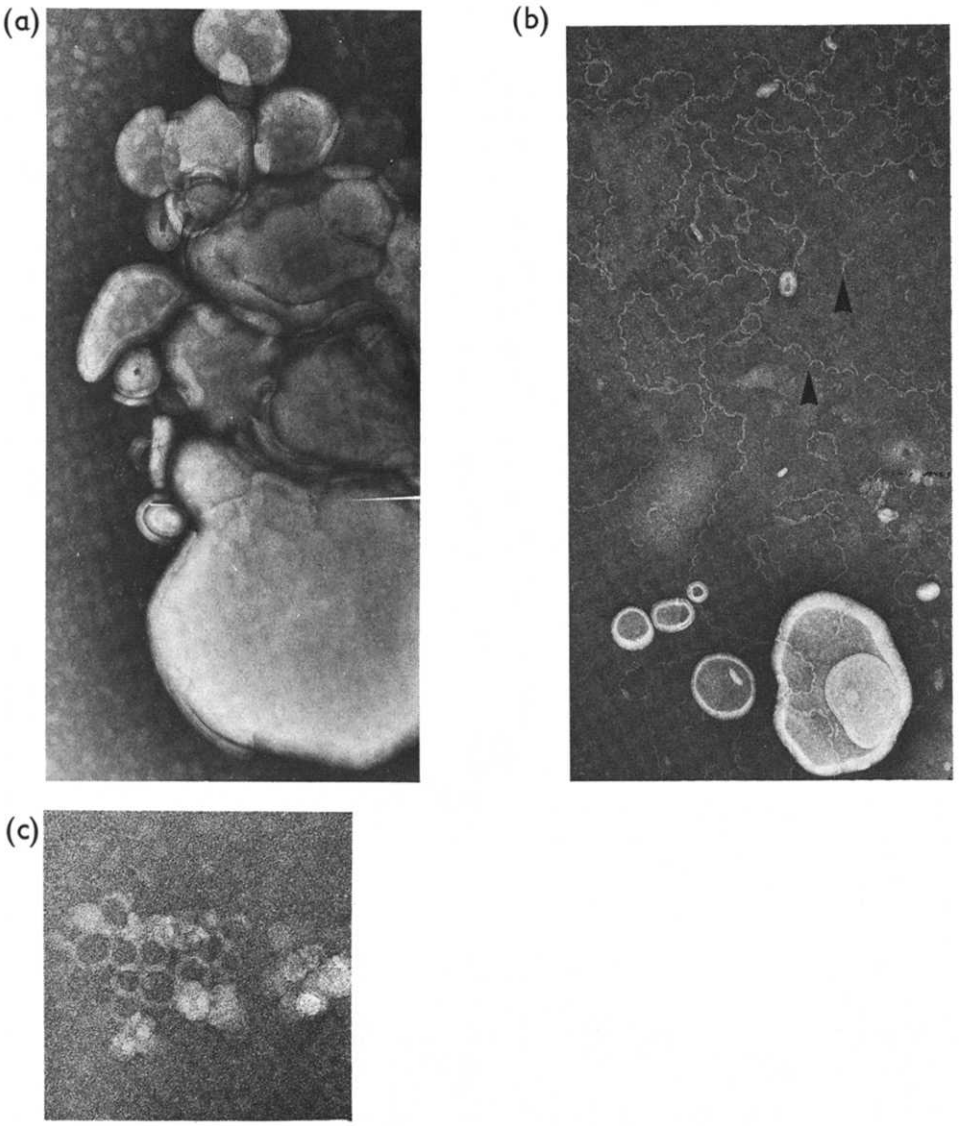


Fig. 6. Ovolecithin dispersion treated with highly purified θ-haemolysin, 50 000 haemolytic units/ml final concentration. (a) Control ovolecithin dispersion in the presence of 0.5 mM β-mercaptoethanol after incubation in Tris buffer for 5 min at 37 °C, magnification × 73 000. (b) Ovolecithin dispersion treated with highly purified θ-haemolysin as under (a). Note the intact lamellae and the long ribbons of arc-shaped structures. These differ in size, 4.1 and 6.8 nm thick (arrow heads), magnification × 73 000. (c) Activated θ-haemolysin, a group of ring-like structures, magnification × 154 000. Rings 32–45 nm outside diameter, 6.5 nm thickness.

(Fig. 6b). However, the lipid bilayers appeared largely unaffected and intact. Areas with what appeared to be "holes" bordered by ring structures such as those seen in haemolysin-treated liposomes (see Fig. 4b) were not observed. These arcs appeared to be of two thickness, the less frequent type being similar to those observed after

treatment of erythrocyte ghosts, mixed lipid dispersions and cholesterol. By contrast, interaction of haemolysin with cholesterol free lecithin dispersions (either vegetable lecithin or synthetic β-γ-dipalmitoyl DL-α lecithin) did not induce the formation of arc or ring shaped structures, and the lecithin bilayers appeared no different from controls.

Both activated and unactivated θ-haemolysin preparations visualised by the negative staining technique revealed arcs or ring structures, similar to those seen in large numbers after interaction of θ-haemolysin with membranes and lipid dispersions (Fig. 6c). It must be emphasised that these structures were present in extremely small numbers and were revealed only after very extensive searching.

Treatment of bacterial membranes with highly purified θ-haemolysin

Plasma membranes of *Micrococcus lysodeikticus*, which have been extensively characterised and lack cholesterol and phospholipids commonly found in mammalian membranes, i.e. lecithin, sphingomyelin and phosphatidylethanolamine [27], were treated with highly purified activated θ-haemolysin. Neither changes in membrane structure nor the presence of ring or arc-shaped structures were seen.

DISCUSSION

In this study two highly purified cytolytic factors produced by *C. perfringens* have been used to evaluate the specificity of some morphological changes in membranes previously ascribed to the action of commercially available phospholipase C [3–13]. The identification of θ-haemolysin as the agent responsible for the formation of ring and arc-shaped structures is based on the following:

(i) Phosphate buffered saline as commonly used by virologists is completely inhibitory to phospholipase C [28], whereas it is the common diluent for O-labile haemolysins [19, 29–31].

(ii) EDTA, used in some instances [9], is an effective inhibitor of phospholipase C and may irreversibly inactivate the enzyme [32, 33], but is non-inhibitory to O-labile haemolysins and may stabilise them [34].

(iii) θ-haemolysin, but not highly purified phospholipase C caused the formation of ring and arc-shaped structures identical to those previously attributed to commercial phospholipase C [3–13].

(iv) The heat lability of θ-haemolysin, its interaction with cholesterol and non-interaction with membranes lacking cholesterol are properties consistent with those ascribed to the unidentified agent of Simpson and Hauser [6] and O-labile haemolysins [14, 15].

(v) The O-labile haemolysins, streptolysin O and cereolysin, cause the formation of holes or pits or ring and arc-shaped structures identical to those described in this study in treated erythrocyte ghosts [10, 35].

These points further substantiate earlier suggestions that θ-haemolysin was the factor involved in commercial phospholipase C [36, 37].

Dourmashkin and Rosse [10] reported that not all the preparations of phospholipase C they tested caused the formation of ring or arc-shaped structures on erythrocyte ghosts. The oxygen-lability of θ-haemolysin in solution and its susceptibility to freezing in dilute solution [38, 39] may well account for the discrepancies in their

observations. It is important to emphasise that θ-haemolysin, unlike streptolysin 0, is produced by *C. perfringens* in the active state and that some of it remains active throughout purification [39] or after freeze-drying of crude material. Moreover, commercially available phospholipase C preparations from *C. perfringens* vary greatly in θ-haemolysin titre when titrated without activation immediately after dissolving e.g. 90 haemolytic units/mg to 10 000 haemolytic units/mg, (Fredholm, B., Möllby, R. and Smyth, C. J., unpublished data) and this may account for other discrepancies in the effects of commercial phospholipase C in the literature [40, 41].

Habermann and Pohlmann [42] described morphological changes in human erythrocyte ghosts treated with highly purified θ-haemolysin which were dependent on the concentration of haemolysin and the ionic environment of the treated ghosts. They did not observe the formation of ring or arc-shaped structures. Other lytic agents known to produce circular structures described as holes or pits in erythrocyte and artificial membrane systems include filipin [43–45], digitonin, alfalfa saponins (medicagenic acid-3-β-D-glucoside) [10, 11] and immune lysis with antibody and complement [10, 46]. Filipin and saponin have properties in common with 0-labile haemolysins: (i) only sterols possessing a 3-β-OH group, a planar sterol nucleus and a hydrophobic group on position 17 of the nucleus are able to interact with these agents, although the alfalfa saponins are not dependent on the 3-β-OH group [47]; (ii) only natural or artificial membranes containing cholesterol are susceptible to these agents [48], and (iii) Shany et al. [49] demonstrated reciprocal interference of binding to natural and artificial membranes between 0-labile haemolysins, saponin and filipin, suggesting a common binding site.

The question arises as to whether these holes or pits extend through the full thickness of the membrane. Freeze-etching data suggests that these features on natural and artificial membranes are not transverse holes in the case of filipin [44, 50] saponin [51] or immune lysis [46]. As the structures seen on treatment of erythrocyte ghosts with θ-haemolysin were released from the membranes, at higher concentrations, they are probably surface located. Freeze-etching studies should provide useful data on this aspect.

Some authors have reported a substructure in the rings or arcs formed after treatment with commercial phospholipase C. However, careful examination of rings at high magnification (Figs 2c, 4c) did not reveal a substructure readily distinguishable from phase contrast effects.

The rare occurrence of arcs and ring structures in highly purified θ-haemolysin preparations raises several important questions concerning their nature and origin. Their presence, albeit in extremely small quantities in purified haemolysin suggests that they may be a polymeric form. Indeed high molecular weight forms of 0-labile haemolysins have been reported for streptolysin 0 [52], listeriolysin [53] and θ-haemolysin [18, 39]. Spontaneous polymerisation, and a more extensive polymerisation induced by lipid, has been reported for the α-haemolysin of *Staphylococcus aureus* [20]. If this explanation is indeed the case, then the abundance of rings and arcs in the reaction mixtures containing cholesterol, and their absence both in reaction mixtures containing cholesterol-free lecithin, or containing bacterial membranes suggests that polymerisation of θ-haemolysin may be specifically induced by cholesterol. A less likely explanation, that the arcs and rings represent a complex of θ-haemolysin with cholesterol would mean that they are formed in the original culture super-

natant fluid from which the haemolysin was purified. Protein/lipid complexes of somewhat similar appearance have been observed after interaction of filipin with cholesterol and cholesterol-containing membranes [54].

To investigate whether phospholipids inhibited θ-haemolysis, twelve commercially available phospholipids, stated to be highly purified or chromatographically pure, were tested. Of five lecithin preparations used at 1 mg/ml final concentration in the haemolytic assay, two were non-inhibitory, one just significantly inhibitory (titre reduced 4-fold) and two clearly inhibitory (titre reduced 2^5–2^6) (Smyth, unpublished data). Similar disparities in inhibitory effects were observed with preparations of other phospholipids. However, because of the sensitivity of θ-haemolysin to inhibition by cholesterol, $\leqslant$ 10 ng/haemolytic units, [18], the inhibitory effects observed with phospholipids may be due to contamination with nanogram amounts of cholesterol e.g. reduction of the θ-haemolysin titre by 2^6 would be effected by 0.64 μg cholesterol/mg lecithin.

It remains to be shown if there is any relationship between ring formation and permeability changes leading to haemolysis of intact erythrocytes. The release of ring structures from treated erythrocytes ghosts and lipid dispersions makes isolation and investigation of their composition possible. The stoichiometry of the interaction of O-labile haemolysins with cholesterol has not been investigated. Physicochemical techniques would be of use in clarifying the nature of their interaction with lipids.

ACKNOWLEDGEMENTS

During part of this work CJS was in receipt of a Science Research Council Studentship; JPA, the Alan Johnston Moseley Research Fellowship of the Royal Society and JHF, was supported by a Medical Research Council Grant. The technical and photographic assistance of Mr Alex Ellis is gratefully acknowledged.

REFERENCES

1 Zwaal, R. F. A., Roelofsen, B. and Colley, M. C. (1973) Biochim. Biophys. Acta 300, 159–182
2 Möllby, R., Nord, C.-E. and Wadström, T. (1973) Toxicon 11, 139–147
3 Padgett, F. and Levine, A. S. (1965) Virology 27, 633–637
4 Simpson, R. W. and Hauser, R. E. (1965) Virology 27, 642–646
5 Simpson, R. W. and Hauser, R. E. (1966) Virology 29, 654–667
6 Simpson, R. W. and Hauser, R. E. (1966) Virology 30, 684–697
7 Kemp, C. L. and Howatson, A. F. (1966) Virology 30, 147–151
8 Schulze, I. T. (1972) Virology 47, 181–196
9 Vernon, S. K., Neurath, A. R. and Rubin, B. A. (1972) J. Ultrastruct. Res. 41, 29–42
10 Dourmashkin, R. R. and Rosse, W. F. (1966) Am. J. Med. 41, 699–710
11 Humphrey, J. H. and Dourmashkin, R. R. (1969) Adv. Immunol. 11, 75–115
12 Benedetti, E. L. and Emmelot, P. (1966) J. Microsc. 5, 645–648
13 Benedetti, E. L. and Emmelot, P. (1968) in The Membranes (Dalton, A. J. and Haguenau, F., eds), Vol. IV, pp. 33–120, Academic Press, London
14 Bernheimer, A. W. (1970) in Microbial Toxins-Bacterial Protein Toxins (Ajl, S. J., Kadis, S. and Montie, T. C. eds), Vol. 1, pp. 183–209, Academic Press, London
15 Bernheimer, A. W. (1974) Biochim. Biophys. Acta 344, 27–50
16 Hauschild, A. H. W. (1971) in Microbial Toxins-Bacterial Protein Toxins (Kadis, S., Montie, T. C. and Ajl, S. J., eds), Vol. IIA, pp. 159–188, Academic Press, London

17 Ispolatovskaya, M. V. (1971) in Microbial Toxins-Bacterial Protein Toxins (Kadis, S., Montie, T. C. and Ajl, S. J., eds), Vol. IIA, pp. 109–158, Academic Press, London
18 Smyth, C. J. (1974) J. Gen. Microbiol. in the press
19 Smyth, C. J. and Arbuthnott, J. P. (1974) J. Med. Microbiol. 7, 41–66
20 Arbuthnott, J. P., Freer, J. H. and Billcliffe, B. (1973) J. Gen. Microbiol. 75, 309–320
21 Möllby, R. and Wadström, T. (1973) Biochim. Biophys. Acta 321, 569–584
22 Freer, J. H., Arbuthnott, J. P. and Billcliffe, B. (1973) J. Gen. Microbiol. 75, 321–332
23 Hoogeveen, J. Th., Juliano, R., Coleman, J. and Rothstein, A. (1970) J. Membrane Biol. 3, 156–172
24 Salton, M. R. J. and Freer, J. H. (1965) Biochim. Biophys. Acta 107, 531–538
25 Brenner, S. and Horne, R. W. (1959) Biochim. Biophys. Acta 24, 103–110
26 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
27 De Siervo, A. J. and Salton, M. R. J. (1971) Biochim. Biophys. Acta 239, 280–292
28 Macfarlane, M. G. and Knight, B. C. J. G. (1941) Biochem. J. 35, 884–902
29 Bernheimer, A. W. and Grushoff, P. (1967) J. Gen. Microbiol. 46, 143–150
30 Shumway, C. N. and Klebanoff, S. J. (1971) Infect. Immun. 4, 388–392
31 Alouf, J. E., Viette, M., Corvazier, R. and Raynaud, M. (1965) Ann. Inst. Pasteur 108, 476–500
32 Ispolatovskaya, M. V. and Klimacheva, L. V. (1966) Biokhimiya 31, 491–500
33 Ispolatovskaya, M. V. (1970) Biokhimiya 35, 434–439
34 Pivnick, H., Habeeb, A. F. S. A., Gorenstein, B., Stuart, P. F. and Hauschild, A. H. W. (1964) Can. J. Microbiol. 10, 329–344
35 Pendleton, I. R., Kim, K. S. and Bernheimer, A. W. (1972) J. Bacteriol. 110, 722–730
36 Smyth, C. J., Arbuthnott, J. P. and Freer, J. H. (1972) J. Gen. Microbiol. 73, xxvi (abstr.).
37 Bernheimer, A. W. (1972) in Streptococci and Streptococcal Diseases; Recognition, Understanding and Management (Wannamaker, L. W. and Matsen, J. M., eds), pp. 19–31, Academic Press, New York
38 van Heyningen, W. E. (1941) Biochem. J. 35, 1257–1269
39 Roth, F. B. and Pillemer, L. (1955) J. Immunol. 75, 50–56
40 Rosenthal, J. W. and Fain, J. H. (1971) J. Biol. Chem. 246, 5888–5895
41 Möllby, R., Wadström, T., Smyth, C. J. and Thelestam, M. (1974) J. Hyg. Epidemiol. Microbiol. Immunol. 18, 259–270
42 Habermann, E. and Pohlmann, G. (1959) Arch. Exp. Pathol. Pharmakol. 237, 487–494
43 Kinsky, S. C., Luse, S. A., Zopf, D., van Deenen, L. L. M. and Haxby, J. (1967) Biochim. Biophys. Acta 135, 844–861
44 Tillack, T. W. and Kinsky, S. C. (1973) Biochim. Biophys. Acta 323, 43–54
45 Bittman, R., Chen, W. C. and Anderson, O. R. (1974) Biochemistry 13, 1364–1373
46 Iles, G. H., Seeman, P., Naylor, D. and Cinader, B. (1973) J. Cell Biol. 56, 528–539
47 Kinsky, S. C. (1970) Annu. Rev. Pharmacol. 10, 119–142
48 Demel, R. A., Cromberg, F. J. L., van Deenen, L. L. M. and Kinsky, S. C. (1968) Biochim. Biophys. Acta 150, 1–14
49 Shany, S., Bernheimer, A. W., Grushoff, P. and Kim, K. S. (1974) Mol. Cell Biochem. 3, 179–186
50 Verkleij, A. J., de Kruijff, B., Gerritsen, W. F., Demel, R. A., van Deenen, L. L. M. and Ververgaert, P. H. J. (1973) Biochim. Biophys. Acta 291, 577–581
51 Seeman, P., Cheng, D. and Iles, G. H. (1973) J. Cell Biol. 56, 519–527
52 Alouf, J. E. and Raynaud, M. (1973) Biochimie 55, 1187–1193
53 Jenkins, E. M. and Watson, B. B. (1971) Infect. Immun. 3, 589–594
54 de Kruijff, B. and Demel, R. A. (1974) Biochim. Biophys. Acta 339, 57–70