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Mechanism of Complement Cytolysis and the Concept of Channel-Forming Proteins [and Discussion]

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Mechanism of complement cytolysis and the concept of channel-forming proteins

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[Plates 1–2]

Complement damages membranes via the terminal reaction sequence that leads to the formation of membrane-bound, macromolecular C5b-9(m) protein complexes. These complexes represent C5b-8 monomers to which varying numbers of C9 molecules can be bound. Complexes carrying high numbers of C9 (*ca.* 6/8–12/16?) exhibit the morphology of hollow protein channels. Because they are embedded within the lipid bilayer, aqueous transmembrane pores are generated that represent the primary lesions caused by complement in the target cell membrane.

Many other proteins damage membranes by forming channels in a manner analogous to the C5b-9(m) complex. Two prototypes of bacterial exotoxins, *Staphylococcus aureus* α-toxin and streptolysin-O, are discussed in this context, and attention is drawn to the numerous analogies existing among these protein systems. Common to all is the process of self-association of the native proteins to form supramolecular complexes. This event is in turn accompanied by a unique transition of the molecules from a hydrophilic to an amphiphilic state.

1. Introduction

Proteins that damage alien cells act either intracellularly or at the level of the plasma membrane. Membrane damage in turn can be incurred either enzymatically (for example, bacterial phospholipases) or on a physical basis. A major mechanism for physical membrane perturbation is the formation of transmembrane pores through protein insertion into the target lipid bilayer. We base the present discussion of this process on data recently obtained for *Staphylococcus aureus* α-toxin, streptolysin-O and the C5b-9(m) complement complex. Since a detailed discussion of the C5b-9(m) complex has recently appeared (Bhakdi & Tranum-Jensen 1983*c*), we will here deal with the concept of membrane damage by channel-formers in broader terms and, in particular, draw attention to many common aspects shared by these protein systems.

The hallmark of the membrane-damaging process by channel-formers is the transition of proteins from a water-soluble state to an amphiphilic state. Through the appearance of lipid-binding surfaces, the proteins are able to enter into and become firmly anchored within the apolar regions of a target membrane. The biochemical basis of this hydrophilic–amphiphilic transition has not yet been elucidated for any single channel-former, but structural studies on several of these proteins are now under way (for example, Biesecker *et al.* 1982). Self-association of native molecules to form supramolecular oligomeric structures appears to provide the driving force for the physical transition of the proteins. Oligomerization is synonymous with the

formation of partly or totally circularized, macromolecular structures that ultimately build the walls of the transmembrane pores. The convex surfaces of these are hydrophobic and carry the lipid-binding sites, whereas the concave surfaces are hydrophilic and thus permit the passage of water and ions. Fully closed, circularized ring structures probably form stable pores lined by protein. However, we believe that partly circularized structures also create functional transmembrane channels by virtue of their lipid-repelling, hydrophilic surfaces that come to lie within the membrane plane. Heterogeneity in the number of subunits comprising the individual channels may give rise to functional heterogeneity of pore size. Marked channel heterogeneity is encountered in the case of streptolysin-O and the C5b-9(m) complement complex, whereas α-toxin pores appear to be quite homogeneous in nature. The amphiphilicity of all these protein complexes is evident from many characteristic features, including non-elutability from unsolubilized membranes (Bhakdi *et al.* 1975), detergent-binding (Helenius & Simons 1975; Bhakdi *et al.* 1978), lipid-binding (Bhakdi & Tranum-Jensen 1978; Bhakdi & Tranum-Jensen 1980; Füssle *et al.* 1981; Bhakdi *et al.* 1984) and the ability to become labelled by apolar photolabels (Hu *et al.* 1981).

All of the oligomerized protein complexes studied to date have, fortunately, been found to be remarkably stable. They withstand not only the action of very high concentrations of non-ionic detergent and deoxycholate, but also resist destruction by proteases at neutral pH (Tranum-Jensen *et al.* 1978; Bhakdi & Tranum-Jensen 1978; Füssle *et al.* 1981). Their isolation from target membranes is therefore usually quite simple – much more so that the isolation of native proteins from serum or from bacterial culture supernatants. All three protein channels to be discussed here were originally isolated from membranes after lysis of target erythrocytes with unpurified toxin or complement components. The general approach has been to treat cells with crude or partly purified toxin preparations or with whole serum, and to subsequently isolate the channels from washed and detergent-solubilized membranes. High concentrations of deoxycholate (250 mM) effects quantitative solubilization of erythrocyte membranes (Biesecker *et al.* 1979), and a single centrifugation of membrane solubilisates through linear sucrose density gradients in a low detergent concentration (for example, 6–10 mM) can then already lead to satisfactory purification of many protein channels (Bhakdi & Tranum-Jensen, 1982; Bhakdi *et al.* 1983*b*, Bhakdi *et al.* 1984). During this procedure, the large channel structures will be separated from other membrane and membrane-bound proteins. Moreover, membrane lipids remain floating in the detergent micelles at the top of the gradients (see Helenius & Simons 1975), so that the channels are recovered in extensively delipidated form. The isolated channels have regularly been found to be very immunogenic, and antisera raised against these proteins can subsequently be used in immunological studies of the oligomerized as well as the native proteins (for example, Bhakdi *et al.* 1978).

The basic requirement for initiating the process of protein oligomerization appears to be the presence of a suitably high concentration of the native proteins. Oligomerization can thus often be induced simply by concentrating purified protein preparations and incubation at 37 °C or at higher temperatures. Spontaneous formation of the protein channels in solution has been observed to occur in this fashion with *S. aureus* α-toxin (Arbuthnott *et al.* 1967), tetanolysin (Rottem *et al.* 1982) and with complement component C9 (Podack & Tschopp, 1982; Tschopp *et al.* 1982). On a membrane target, and at lower, physiological concentrations, membrane 'receptors' or specific binding sites probably serve the important function of creating the required local protein concentration. In the case of -SH activated bacterial toxins such as

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streptolysin-O, this function is served by membrane cholesterol (Alouf 1980). In the case of C9, which is the major channel-forming subunit of the complement system, the function is served by membrane-bound C5b-8 (Kolb *et al.* 1972; Kolb & Müller-Eberhard 1974; Podack *et al.* 1982). Such specific binding sites may not be a general requirement for binding of other channel formers, however. Analogous 'receptors' have for instance not been identified for *S. aureus* α-toxin, or for the 'pre-terminal' C5b-7 complement complex.

Protein channels have been characterized on a functional basis by determining the appearance of functional pores in the membrane. Many experimental systems have been used for this purpose, including measurements of release of intracellularly trapped markers (Giavedoni *et al.* 1979; Füssle *et al.* 1981; Ramm & Mayer 1980), and conductance measurements across planar lipid bilayers (Michaels *et al.* 1976). Generally, the different experimental approaches have all yielded data compatible with the concept of true transmembrane pore formation (see Mayer 1978; Bhakdi & Tranum-Jensen 1983*c*). Minor discrepancies arising with regard to the true dimensions of the lesions have probably originated from the different experimental conditions selected by the different groups of investigators, and from the interpretational difficulties inherent in many such systems (for discussion, see Bhakdi & Tranum-Jensen 1983*c*).

2. *S. aureus* α-toxin and streptolysin-O: two prototypes of channel-forming bacterial toxins

(*a*) *S. aureus α-toxin*

S. aureus α-toxin is produced by most pathogenic strains of staphylococci and is considered one of the major factors of staphylococcal pathogenicity (McCartney & Arbuthnott 1978). The toxin is secreted as a hydrophilic, 3.3 *S* monomer of M_r 34000 (Bhakdi *et al.* 1981) with an isoelectric point of approximately 8.5. Upon contact with an appropriate target membrane, the monomers self-associate to form small channels that appear to represent a homogeneous population of 11–12 *S* hexamers (M_r 200000; Bhakdi *et al.* 1981). The latter bind lipid and detergent. The generate circumscribed functional 'holes' in the membrane of resealed erythrocyte ghosts whose effective diameter appears to be 2–3 nm (Füssle *et al.* 1981). In the electron microscope, the channels indeed display a central pore of these dimensions (figure 1, plate 1). The external diameter of the hexamers measures 8.5–10 nm as determined on the extramembranous portion of the cylinder. The hexamer extends approximately 4 nm into the extramembranous phase, so that the volume of the extramembranously orientated portion of the complex can be estimated to be approximately 200–250 nm^3, which already corresponds to a mass of 160000–200000 Da. These calculations indirectly indicate that the intramembranous domain of the α-toxin rings may make up only a small part of the total mass; the walls of the pore within the membrane have not been directly seen by electron microscopy and may in fact be much thinner than those forming the walls of the externally oriented cylinder. These observations serve to illustrate the potential difficulties that may be encountered during future attempts to delineate the architecture of the pore at a molecular level as the amino acid sequence of the protein becomes known.

Triggering of the hexamerization process is not recognizably dependent on the presence of a biochemically defined membrane binding site (Freer *et al.* 1968; Füssle *et al.* 1981). Spontaneous hexamer formation occurs in the absence of a lipid bilayer when purified toxin

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is exposed to specific heating conditions (Arbuthnott *et al.* 1967) or to deoxycholate detergent (Bhakdi *et al.* 1981). Hexamers also form when toxin molecules come into contact with human plasma low density lipoprotein (l.d.l.), probably through unspecific triggering of the oligomerization process by lipid contained in the l.d.l. particle (Bhakdi *et al.* 1983*a*). As with all other channel formers, toxin oligomers are haemolytically inactive, probably owing to their low solubility in water. Binding and hexamer formation of α-toxin on l.d.l. thus causes toxin inactivation and may represent a significant non-immune defence mechanism of the host towards this toxin.

(*b*) *Streptolysin-O*

This toxin is a secreted product of group A β-haemolytic streptococci and represents the prototype of -SH activated bacterial cytolysins (Alouf 1980). At least 14 other bacterial exotoxins belong to this group and share the following properties. All are reversibly inactivated by atmospheric oxygen. The initial binding of toxin is to membrane cholesterol, and membrane damage can thus be produced in a very wide variety of mammalian cells. In contrast, bacterial membranes that lack cholesterol are not attacked by the toxins. All toxins of this class consist of a single polypeptide chain with individual molecular masses ranging from 40000–80000 Da. After binding to cholesterol, toxin molecules self-associate in an apparently temperature-dependent process to form very large, curved rod structures (Duncan & Schlegel 1975) that lie embedded within the bilayer and generate channels.

We have recently isolated streptolysin-O from bacterial culture supernatants and identified two haemolytically active forms (unpublished data). Native toxin has a M_r of approximately 69000 and an isoelectric point of approximately 6.0. During purification procedures, it can be cleaved proteolytically to yield a haemolytic 57000 Da polypeptide with an isoelectric point of approximately 7.4. Both toxin forms bind to erythrocyte membranes yielding the characteristic channel structures. Oligomers of streptolysin-O exhibit marked heterogeneity, and a broad array of structures ranging from C-shaped curved rods to fully closed rings is observed (figure 1). These complexes insert into the membrane to generate very large, 30–35 nm defects in the bilayer. Functional studies also reveal the existence of large hydrophilic pores across such membranes (Buckingham & Duncan 1983). The channels can be particularly well seen after their re-incorporation into bilayers of pure egg lecithin (figure 1). When C-shaped oligomers become membrane-incorporated in such a system, the ensuing channels appear lined only in part by protein (streptolysin-O molecules; figure 1 and Bhakdi *et al.* 1984). Facing the concave sides of the protein channel, a sharp edge of lipid appears to line the residual part of the channel. Insertion of frankly 'incomplete' channels consisting of C-shaped toxin oligomers thus already appears to create transmembrane pores, probably because of the repelling of lipid by the hydrophilic concave sides of the membrane-inserted complexes. It will be of great interest to determine eventually the state of molecular packing and orientation of lipids in such membrane regions.

The fact that isolated and extensively delipidated toxin oligomers can re-associate with pure lecithin serves to underline the fact that cholesterol, although important in triggering the oligomerization process, does not itself significantly contribute to the structure of the channel. Once exposed, the apolar regions of the protein complex also exhibit no recognizable specificity with regard to the type of lipid that can be bound (Bhakdi *et al.* 1984).

Initial estimates yield volumes of streptolysin-O oligomers ranging from approximately

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2000–6000 nm³, corresponding to a range of mass of approximately $1.5–5.0 \times 10^6$ Da. This corresponds to a heterogeneous population of complexes containing approximately 25–80 molecules of native toxin.

3. The cytolytic C5b-9(m) complement complex

The C5b-9(m) complex (the suffix 'm' is used to denote the membrane location of the molecule) forms on and in a membrane after initiation of both classical and alternative complement pathways (figure 2), and is able to damage a wide range of biological membranes,

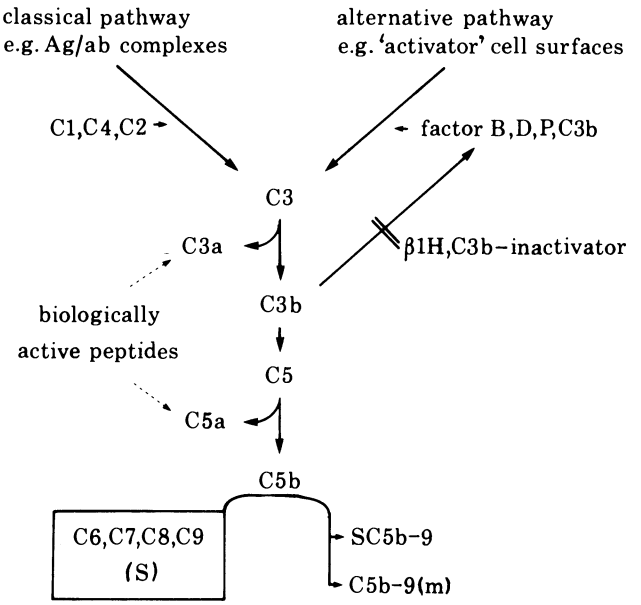

Figure 2. Schematic representation of the major reaction pathways of the complement system.

spanning the scale from prokaryotes to mammals. Although C5b-9(m) is the most complex protein channel recognized at present, it was the first to be isolated and characterized at a biochemical, immunological and ultrastructural level. Very simple methods were first used to demonstrate its existence on target erythrocyte membranes, and to reveal its similarity to integral membrane proteins (Bhakdi *et al.* 1975; figure 3). The complex can easily be isolated in quantity from target erythrocytes without the use of purified components, special reagents or techniques (Bhakdi *et al.* 1983*b*). Essentially, antibody-coated sheep or native rabbit erythrocytes are lysed with an excess of whole human serum, the membranes are washed and solubilized with 250 mM deoxycholate, and the C5b-9(m) complex isolated by a single ultracentrifugation in a detergent-containing sucrose density gradient. The complex sediments to cover a broad region covering 25–40 *S* (apparent sedimentation coefficient). The individual subunits of the complex are readily discerned by SDS-polyacrylamide gel electrophoresis (figure 4). Rabbit antisera raised against the purified complex react with native C5–C9 and with the C5b-9 complex (Bhakdi *et al.* 1978). Moreover, the complex carries characteristic neoantigenic determinants that permit its immunological differentiation from native C5–C9 components (Kolb & Müller-Eberhard 1975; Bhakdi *et al.* 1978; Bhakdi *et al.* 1983*b*). Isolated

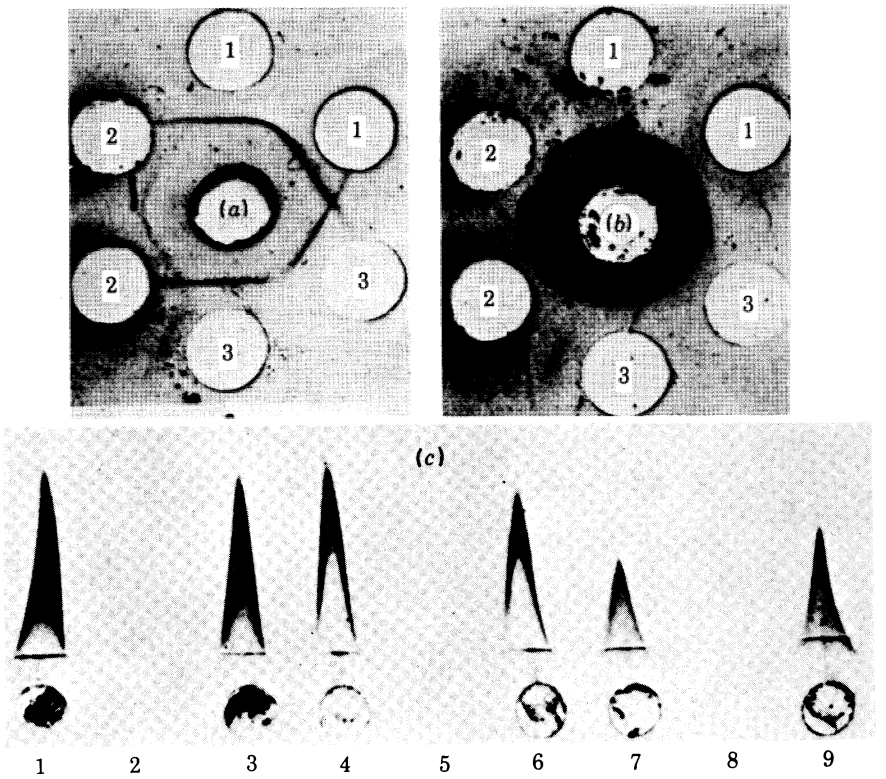

FIGURE 3. (*a*) and (*b*). Original demonstration of the presence of C5b-9 complexes on target erythrocyte membranes (from Bhakdi *et al.* 1975). Membranes were lysed with whole human serum, solubilized with Triton X-100 and analysed by double diffusion using anti-C5 (wells 1), anti-C6 (wells 2) and anti-C9 (wells 3, plate *b*). Fresh human serum was analysed in parallel as the control (plate *a*). Note the reaction of non-identity of native C5, C6 and C9 components (*a*), but the coalescence of the respective precipitates derived from membrane solubilisates (*b*) indicative of complex formation between these proteins, which had previously been identified as the membrane-damaging entities of the complement system (Lachmann & Thompson 1970; Thompson & Lachmann 1970). (*c*) Original demonstration that membrane-bound C5b-9(m) mimics intrinsic membrane proteins and is not eluted from intact membranes (from Bhakdi *et al.* 1975). Complement-lysed erythrocyte membranes were eluted by different salt solutions and the aqueous supernatants applied to wells 2 (elution with dilute EDTA), 5 (elution with 100 mM EDTA), and 8 (elution with 1.2 M NaCl). Membrane pellets before (wells 1, 4, 7) and after elution (wells 3, 6, 9, respectively) were applied in the same plate. Note the entire absence of C5b-9(m) in the salt supernatants as revealed by rocket immunoelectrophoresis with anti-C9. C5b-9(m) was quantitatively recovered in the pellet in every case (equal heights of rocket over wells 3, 6 and 9 compared to 1, 4 and 7, respectively).

DESCRIPTION OF PLATE 1

FIGURE 1. (*a*) Negatively stained fragment of rabbit erythrocyte lysed with *S. aureus* α-toxin. Numerous 10 nm ring-shaped structures are seen over the membrane (arrows). (*b*) Isolated α-toxin hexamers in detergent solution. (*c*) Lecithin liposomes carrying re-incorporated α-toxin hexamers. The hexamers are seen as stubs along the edge of the liposomal membrane and as rings over the membrane (arrows). Characteristically, liposomes that escape incorporation of the toxin are impermeable to the stain. (*d*) Negatively stained, erythrocyte membrane lysed by streptolysin-O showing numerous 25–100 nm long and approximately 7.5 nm broad, curved rods of 13–16 nm inner radius of curvature. Most rods are approximately semicircular, often joined in pairs at their ends. Dense accumulations of stain are seen at the concave side of the rods. When these do not form closed profiles, the stain deposit is partly bordered by a 'free' edge of the erythrocyte membrane (arrows). (*e*) Negative staining of isolated streptolysin-O oligomers, showing numerous curved rod structures identical to those found on toxin-treated membranes. (*f*) Purified streptolysin-O complexes re-incorporated into cholesterol-free lecithin liposomes. The toxin oligomers form holes in the liposomes. Part of the circumference of such holes appear bordered by a 'free' edge of liposomal membrane (unlabelled arrows). p, A lesion seen in profile. Scale bars indicate 100 nm in all frames. Sodium silicotungstate was used as negative stain in frames (*b*)–(*f*). Uranylacetate was used in (*a*).

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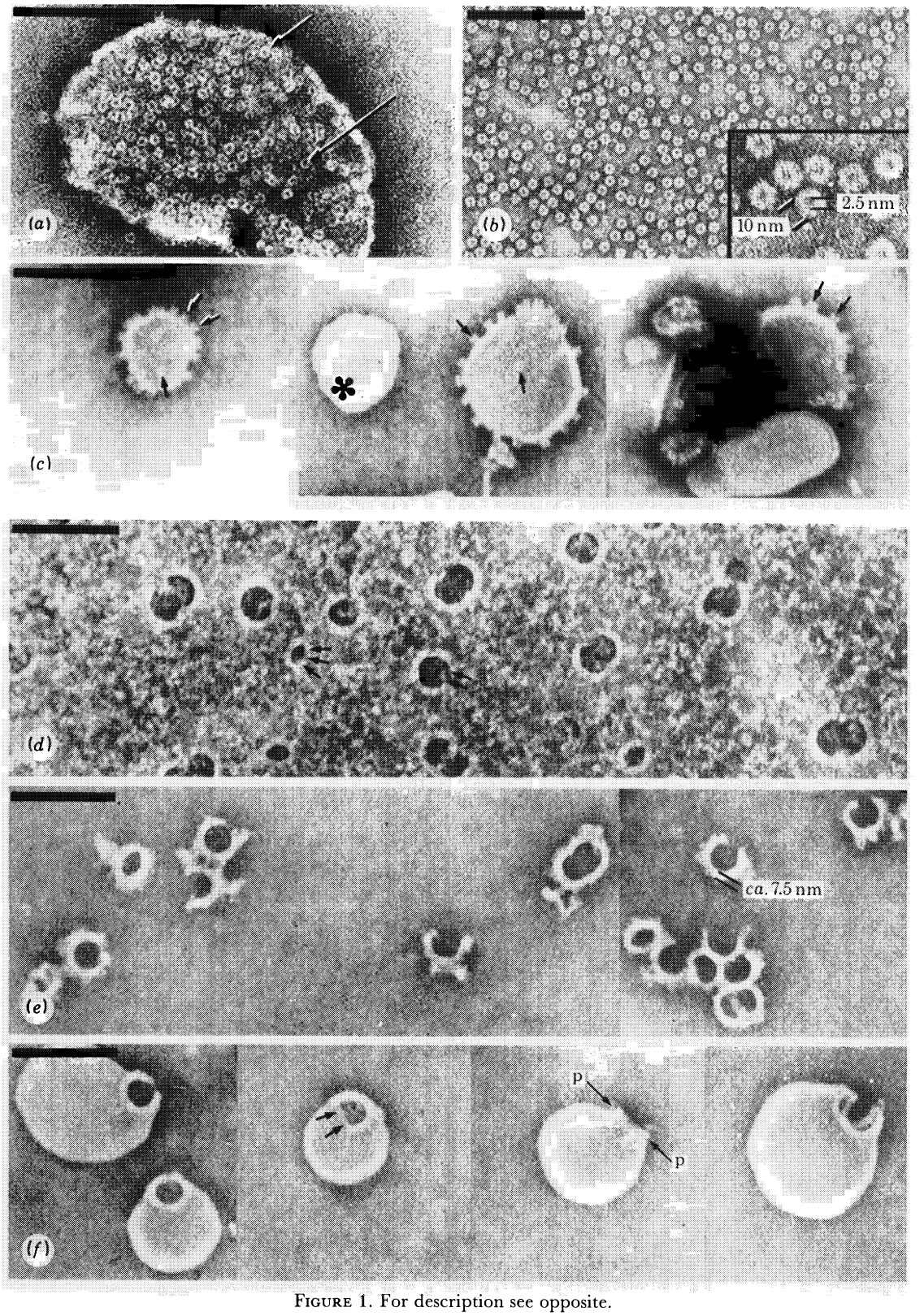

FIGURE 1. For description see opposite.

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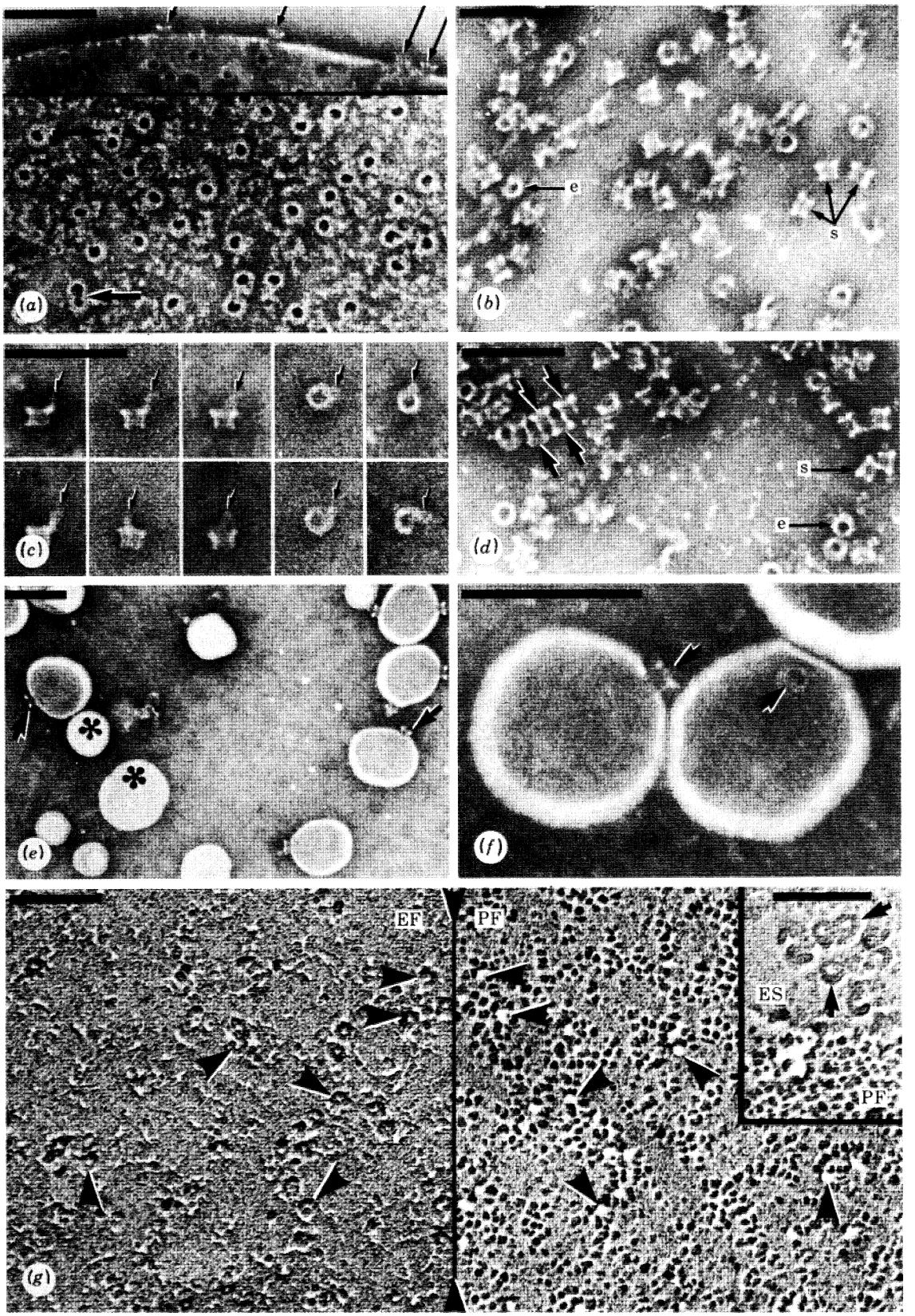

FIGURE 5. For description see opposite.

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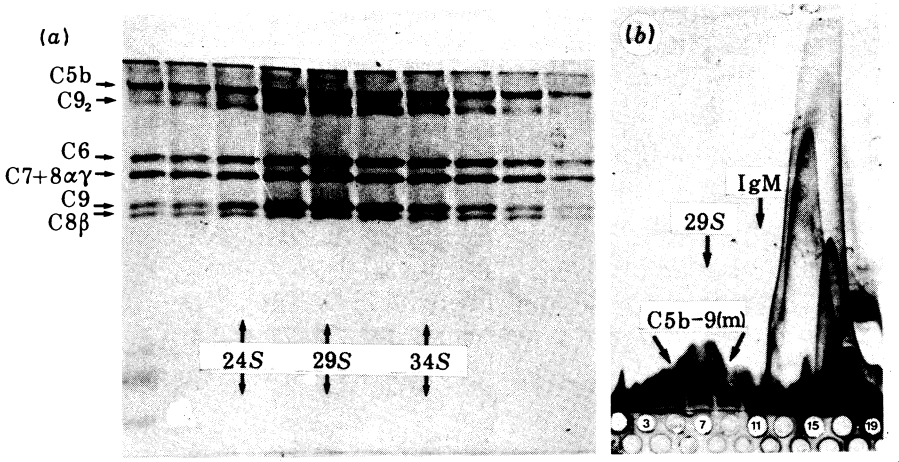

FIGURE 4. Isolation of C5b-9(m) from target erythrocyte membranes be sedimentation in detergent-containing sucrose density gradients. Washed membranes were solubilized with 250 mM deoxycholate and centrifuged in 10–43% (by mass) sucrose density gradients containing 6.25 mM detergent. Twenty fractions were collected and analysed by SDS-polyacrylamide gel electrophoresis (plate *a*) and by fused rocket immunoelectrophoresis with the use of polyspecific antibodies to human serum proteins (plate *b*). Plate *a* shows the gel electrophoresis pattern obtained with the first 10 fractions from the gradient (corresponding to 25–40*S*). The C5b-9(m) complex exhibits a typical polypeptide composition and the individual subunits of the complex have been identified. Direction of sedimentation: right to left.

C5b-9(m) recovered from sucrose density gradients can be directly used in membrane reconstitution studies and studied in the electron microscope.

Since complement components circulate freely in plasma, it is essential that regulatory mechanisms exist to prevent destruction of innocent cells. Major regulatory mechanisms operate at the level of C3/C3b, and the complement cascade may be arrested if C3b-inactivating mechanisms prevail to save the cell from assault by C5b-9(m) (Fearon 1979; Fearon 1980;

DESCRIPTION OF PLATE 2

FIGURE 5. (*a*) Negatively stained, complement-lysed erythrocytes. C5b-9(m) complexes are seen as numerous circular ('classical' lesions over the membrane together with some 'twinned' forms (bold arrows). The C5b-9(m) complexes are seen as 10 nm high cylindrical projections along the bent edge of the ghost membrane at the top (arrows). The light rim representing the sharply bent membrane in tangential view is attenuated or interrupted at the site of attachment of the complexes. (*b*) Negatively stained preparation of isolated C5b-9(m) complexes in detergent solution. The complex has the basic structure of a 15 nm high, thin-walled cylinder, rimmed by an annulus at one end. The cylinder is seen in various levels of tilt between side views (s) and axial projection (e). (*c*) Selection of C5b-9(m) complexes exhibiting a small appendage (arrows), often seen on the annulus, particularly by low electron dose image recording. This stalk carries antigenic determinants of C5 and C6 (Tschopp *et al.* 1982*b*). (*d*) 'Poly-C9' formed by prolonged incubation of purified human C9 in detergent-free buffer solution at 37 °C as described by Tschopp *et al.* (1982*a*). The C9 oligomers exhibit a cylindrical structure closely resembling the C5b-9(m) complex except for the absence of appendages on the annulus. Occasionally, small ordered arrays of cylinders are seen (arrows), associated at the putative apolar terminus opposite the annulus. (*e*) and (*f*) C5b-9(m) complexes (arrows) generated on erythrocytes, purified and re-incorporated into phosphatidylcholine liposomes. Vesicles that escaped incorporation of a complex (asterisks) are characteristically impermeable to the stain. Typically, the complexes project 10 nm exterior to the plane of the membrane. (*g*) Complementary freeze-etch replicas of antibody-sensitized sheep erythrocyte, lysed with human complement. Fracture E-faces (left frame) exhibit numerous ring-shaped structures, interpreted to represent the intramembranous portion of C5b-9(m) cylindrical complexes. The rings are complementary to circular defects in the lipid plateau of the inner membrane leaflet (PF-face). A number of complementary lesions are labelled by arrowheads. Inset at upper right shows C5b-9(m) annuli (arrows) on the etched true outer surface (ES) of a proteolytically stripped ghost membrane. 25° rotary shadowing with Pt. Scale bars indicate 100 nm in frames (*a*)–(*g*). Sodium silicotungstate was used for negative staining in frames (*a*)–(*f*).

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Kazatchkine & Nydegger 1982). If inactivation of cell or particle-bound C3b does not occur to the necessary extent, C5 will be cleaved to its active derivative C5b. No further proteolytic cleavage of terminal C6–C9 components has been identified after this stage of the terminal reaction. C6 and C7 spontaneously associate with C5b to form a trimolecular C5b-7 complex (Kolb & Müller-Eberhard 1972). If this complex forms in a hydrophilic phase, for example, through complement activation in whole serum by immune complexes or alternative pathway activators, the nascent complex will be inactivated through binding of the serum 'S'-protein (Podack & Müller-Eberhard 1978); this may represent the last regulatory mechanism in the entire complement sequence. SC5b-7 will subsequently bind C8 and C9 so that a cytolytically inactive, water-soluble SC5b-9 complex forms whose biological functions, if any, remain unknown at present (Kolb & Müller-Eberhard 1975*b*; Bhakdi & Tranum-Jensen 1983*c*; Bhakdi & Roth 1981).

C5b-7 forming on a target lipid bilayer will, in contrast, insert into the membrane and escape inactivation by the S-protein. It is thus at this stage that hydrophobic regions first appear on a terminal complex (Hammer *et al.* 1975; Hu *et al.* 1981). C5b-7 insertion does not, however, create membrane instability or leakiness (Michaels *et al.* 1976). The complex possesses one binding site (Kolb & Müller-Eberhard 1972) for the β-chain of C8 (Monahan & Sodetz 1981). Binding of C8 appears to create small channels in the bilayer (Michaels *et al.* 1976), and erythrocytes carrying C5b-8 complexes have been reported to lyse slowly (Stolfi 1968). The functional dimensions of C5b-8 channels in erythrocyte membranes have been estimated to be of the order of 0.9 nm (Ramm *et al.* 1982*b*). The ultrastructure of neither C5b-7 nor C5b-8 has been definitely established.

The C5b-8 complex serves as the binding substrate for C9 (Kolb & Müller-Eberhard 1974; Podack *et al.* 1982). Initial binding of one C9 molecule to C5b-8 probably triggers an autocatalytic reaction leading to further attachment and self-association of C9 molecules within the complex. This causes 'widening' of the pore to a maximum of 5–7 nm (Michaels *et al.* 1976; Giavedoni *et al.* 1979; Ramm & Mayer 1980). At a certain, as yet undefined ratio of C9:C5b-8 molecules, defined channel structures are seen in the electron microscope. Analogies may here be drawn to the event of channel formation by –SH activated toxins: C5b-8 serves as the initial receptor triggering the C9-oligomerization reaction much as cholesterol serves to bind initially streptolysin-O. Formation of ultrastructurally visible channels *per se* is then the consequence of C9-oligomerization in the former, and of toxin molecules in the latter case. By analogy to spontaneous oligomerization occurring with -SH activated toxins (as reported for tetanolysin, by Rottem *et al.* (1982)) and *S. aureus* α-toxin under specific conditions (Arbuthnott *et al.* 1967), isolated C9 will also spontaneously oligomerize to form the channel structures characteristic of C5b-9(m) complexes carrying high numbers of C9 (Podack & Tschopp 1982; Tschopp *et al.* 1982). These channel structures have been described as hollow protein cylinders rimmed by an annulus at one terminus harbouring an internal pore of 10 nm. The cylinders are vertically orientated to the membrane plane and, when viewed on the face on the membrane surface, are seen as the typical ring structures that were originally described by Humphrey & Dourmashkin (1969) as the classical complement 'lesions'. Membrane reconstitution studies revealed that 4–5 nm of the thin-walled portion of the cylinder distal to the annulus carry the lipid-binding sites which insert into the membrane. Frank interruptions in the continuity of the bilayer are observed at the sites of insertion of the complex, and stain deposits are seen traversing the internal diameter of the channel to enter liposomes (figure 5, plate 2; Bhakdi

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& Tranum-Jensen 1978). The micromorphology of such protein cylinders embedded within the bilayer of target erythrocytes has recently been studied by freeze–fracture electron microscopy (Tranum-Jensen & Bhakdi, 1983). The originally proposed model for fully formed C5b-9(m) complexes (Tranum-Jensen *et al.* 1978; Bhakdi & Tranum-Jensen 1978) was fully corroborated using this technique. In particular, complementary replicas showed that each EF-face ring corresponded to a hole in the lipid plateau of the PF-face, and etched fractures confirmed the existence of a central, water-filled pore in the molecule (figure 5; Tranum-Jensen & Bhakdi 1983). Today, there can be little doubt that these C5b-9(m) channels represent the primary membrane lesion of complement.

It is now recognized that the bulk of the described cylindrical structure is composed of C9 oligomers (Tschopp *et al.* 1982). The structure and exact orientation of C5b-8 within such C5b-9(m) remains unclear. Determinants of C5 and C6 are present on a 'stalk' structure projecting from one side of the C9 'polymer' channel (Tschopp *et al.* 1982; figure 5). Because there is only one stalk on each 'poly-C9' cylinder, each complex is a monomer with respect to C5b-8 (Tschopp *et al.* 1982; Tschopp 1983). It seems to us likely that the stalk structure represents but a part of C5b-8, the other participating in formation of the channel walls, but obscured as such by the predominating C9 structure.

Recent functional and biochemical studies have shown that C5b-9(m) complexes generated by the action of whole human serum on erythrocytes comprise heterogeneous populations with respect to C9 content (Boyle *et al.* 1979; Boyle *et al.* 1981; Ramm *et al.* 1982; Bhakdi & Tranum-Jensen 1984; Tschopp 1983), and the typical cylindrical structure of C5b-9(m) discussed above is characteristic only of these complexes carrying relatively high numbers of C9 molecules. A major cause of C5b-9(m) heterogeneity is a naturally occurring, relative shortage of C9 in serum (Bhakdi & Tranum-Jensen 1984). Thus, serum concentrations of both C8 and C9 are in the range of 70–80 μg ml^{-1}, equivalent to two molecules C9 for each molecule C8. At high cell concentrations and excess of C5b-8 membrane binding sites relative to applied serum dosage, low C9:C8 average binding ratios ensue (C9:C8 = 2–3:1 on the membrane). As serum doses given to a constant number of target cells are increased, more and more C9 molecules are free to bind to C5b-8 and higher C9:C8 ratios are found. SC5b-9 formation does not occur as a bystander reaction so that no competition for C9 takes place in the fluid-phase with erythrocytes as target cells. At very high serum concentrations (for example, 5–10 ml whole serum given to 10^9 cells), a maximal average of six to eight molecules of C9 per cell-bound C8 is found. Is has been reported that a completely built C9 channel formed by purified C9 in the absence of C5b-8 has a mass of approximately 10^6 and is composed of 12–16 molecules of C9 (Tschopp 1983). One such channel bound to a C5b-8 monomer would yield a C5b-9(m) complex with the subunit composition $(C5b\text{-}8)_1C9_{12}$ with a M_r of $1.5\text{–}1.6 \times 10^6$. This probably represents the maximum M_r of a completely formed, unit lesion of normal dimensions (Tschopp 1983). Since a maximum average C9:C8 ratio of only 6–8:1 is found in C5b-9(m) preparations, however, it is apparent that such 'complete' complexes can only represent a minority of the entire population. On the target membrane, an array of other complexes exist with compositions probably covering the range $(C5b\text{-}8)_{1\text{–}2} \longrightarrow (C5b\text{-}8)_1C9_{12}$. Close scrutiny of individual ring-shaped lesions on the surface of target erythrocytes indeed reveals that most complexes exhibit defects of closure (figure 5; Tranum-Jensen & Bhakdi 1983; Tschopp 1983). Although such complexes probably harbour fewer than the maximum of 12 C9 molecules, they would still exhibit the typical cylindrical structure when viewed in profile. Furthermore, many

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complexes exhibit aberrant forms, rings of smaller diameter (probably also containing fewer C9 molecules) abound within any random population of C5b-9(m) complexes, and even under conditions of C9 excess a large number of terminal complexes of ill-defined morphology exist on an erythrocyte membrane. It is not known which number of C9 bound to C5b-8 is critical for the appearance of the ultrastructurally defined cylinder forms, nor is it clear at which C9:C8 ratio the largest (maximum) functional pore (5–7 nm) appears. At present, the collective data suggest that the majority of channel-like structures has the composition $(C5b\text{-}8)_1C9_{6-9}$ corresponding to a M_r range of 1×10^6–1.3×10^6. A dimer nature with respect to C5b-8 has been claimed in the past (Podack *et al.* 1980; Podack & Müller-Eberhard 1981; Podack *et al.* 1982), but since our initial criticism of these data was voiced (Bhakdi & Tranum-Jensen 1981), other functional (Ramm *et al.* 1982*a*) and structural studies have appeared (Tschopp *et al.* 1982; Tschopp 1983) that clearly speak against the dimer model. We adhere to our original proposal that the unit lesion is a monomer with respect to C5b-8, and believe that the earlier structural data taken to support the dimer model (Podack *et al.* 1980) now require revision and re-investigation. The concept of lesion heterogeneity deriving from differing numbers of C9 molecules attached to C5b-8 monomers also is in contrast to the model of heterogeneity owing to the parallel existence of monomer and dimer complexes as proposed by Podack *et al.* (1982). However, our data and model are in complete accordance with those of Tschopp (1983).

4. Membrane damage by channel formers

A relatively simple chain of events can be envisaged to underlie the process of membrane penetration by channel-forming proteins. The initial reaction leading to formation of the first oligomers (dimers?) is a process obviously exhibiting very slow kinetics in a hydrophilic environment. Once formed, however, these oligomers trigger a second, autocatalytic reaction that is fast, leading to generation of the channels. Crucial to the triggering event on a biological membrane is the build-up of a high local concentration of the protein, this being necessary to initiate the first oligomerization process. Specific binding substrates such as cholesterol for -SH activated toxins, and C5b-8 for C9 optimally fulfil this function. The mechanisms responsible for protein binding in the absence of such identifiable membrane receptors are not understood. However, it appears likely that quite unspecific factors such as surface membrane charge play important roles. It is of interest to note that the binding of α-toxin to human erythrocytes displays marked pH-dependence, and binding at low pH (5.5) increases by an order of magnitude compared with binding at pH 7.0 (unpublished observations). This is probably due to neutralization of membrane surface charge at low pH. A related phenomenon may be the differential binding of pre-formed 'poly-C9' cylindrical complexes in a hydrophilic phase to liposomal membrane bilayers, but not to erythrocyte membranes (Tschopp *et al.* 1983). Since C9 itself has a low pI (around pH 4.6; Bhakdi *et al.* 1976), its high negative charge at neutral pH would prevent its binding to the erythrocyte surface. Interestingly, all channel-forming bacterial toxins have relatively high isoelectric points (pH 6–8) and this would facilitate their initial diffusion to the surface of biological cell membrane targets.

Membrane insertion (penetration) *per se* may be a spontaneous process driven simply by the energetically favoured hydrophobic–hydrophobic interactions. With regard to the mode of pore formation, two basic mechanisms may be envisaged. First, lipid (and membrane proteins) may be expelled at the channel sites. Alternatively, a 'force-aside' mechanism could cause lateral

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displacement of membrane constituents. In the latter case, there would be no necessity for expulsion and release of integral membrane proteins and lipids into the environment. Although the former model for channel formation has been favoured in the past (for discussion, see Bhakdi & Tranum-Jensen 1983*c*), recent data from our laboratory tend to support the latter (unpublished data). The repelling of lipid (and membrane protein) may be initiated by the insertion of 'incomplete', non-circularized oligomeric structures with exposure of hydrophilic surfaces within the membrane plane, such as appears to occur with the majority of streptolysin-O complexes. Complete circularization of the complexes is thus probably not even necessary for generation of functional transmembrane pores. These arguments probably also hold for C5b-9(m) complexes, where generation of lesion heterogeneity through differential binding of C9 shows obvious analogies to heterogeneity of the streptolysin-O channels. Although there are uncertainties regarding the exact architecture of the pores, all the data basically support the channel model of membrane damage as originally proposed by Mayer for the C5b-9 complex (Mayer 1972).

5. Concluding remarks

The concept of membrane damage by channel-forming proteins today appears quite well documented on a functional, biochemical and ultrastructural basis, and it is to be expected that many other protein systems will eventually be found to operate in a similar fashion. It is easy to anticipate that cell biological and cell physiological aspects will now become a most fruitful field to explore. Obvious problems relate to the fate of channels after their attachment to nucleated cells, and to possible secondary pathophysiological effects mediated by the proteins. Progress in the study of these phenomena will lead us to appreciate more fully the true biological significance of membrane damage by these interesting proteins.

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Discussion

C. A. Pasternak and K. J. Micklem (*Department of Biochemistry, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K.*). Dr Bhakdi has shown that the transmembrane channels formed by the C5b–C9 complement proteins resemble the channels formed by two types of bacterial toxin (*S. aureus* α-toxin and streptolysin O) in several regards. We wish to draw attention to the fact that haemolytic Sendai virus (Poste & Pasternak 1978) or influenza virus at pH 5.3 (Patel & Pasternak 1983) form transmembrane channels that share certain characteristics with the channels formed by complement or by bacterial toxins (Pasternak & Micklem 1983). Because virally induced channels can be modulated by external Ca^{2+} (Poste & Pasternak 1978), it would seem profitable to test the Ca^{2+} sensitivity of channels formed by complement and bacterial toxins also.

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S. Bhakdi. The possible similarities between membrane perturbation induced by viral membrane components and channel-forming proteins are an interesting point that would be worth investigating in the future. It is noteworthy that membrane-binding and channel-formation by C5b-9, and by the bacterial toxins discussed at this meeting, are calcium-independent processes.

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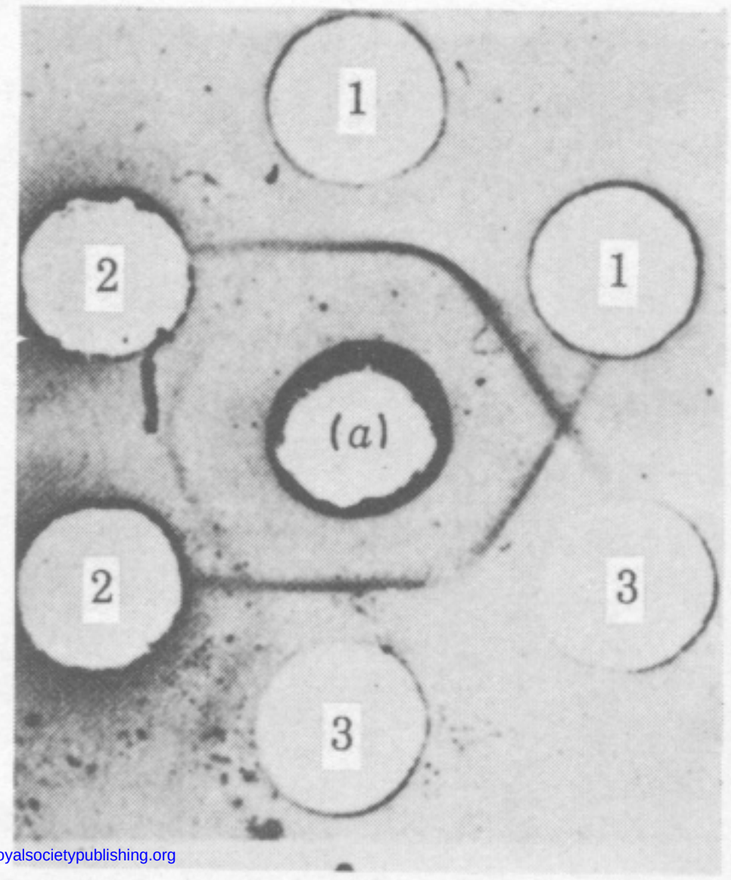
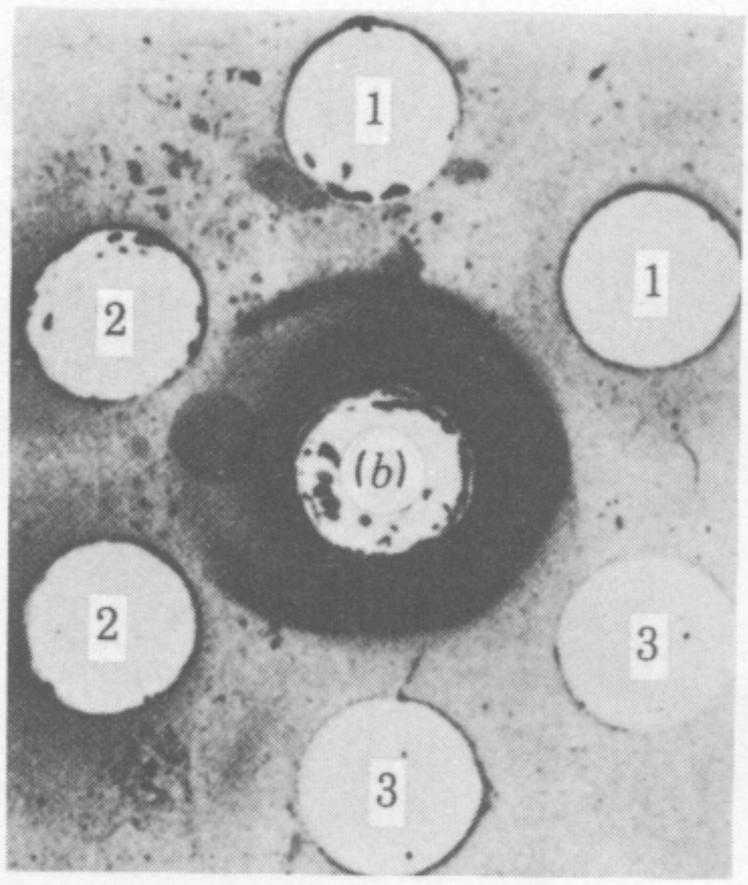




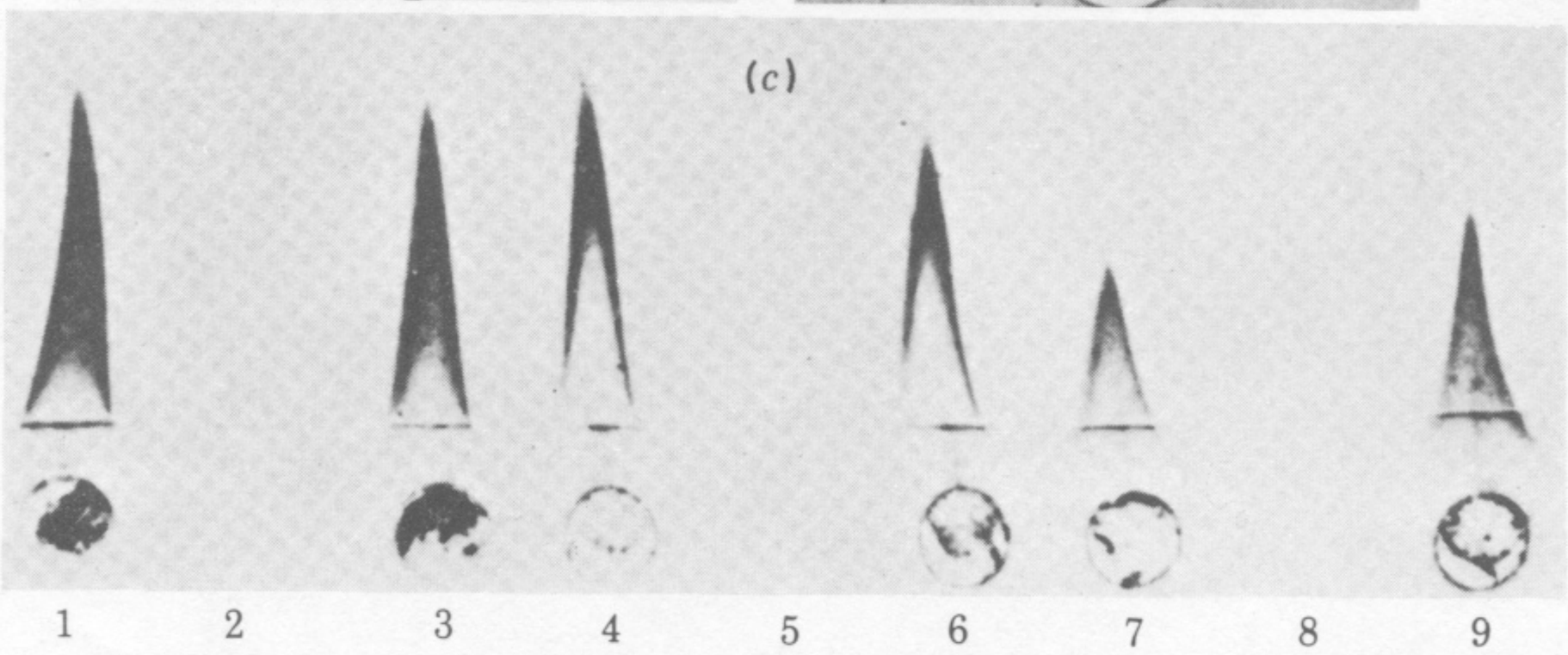
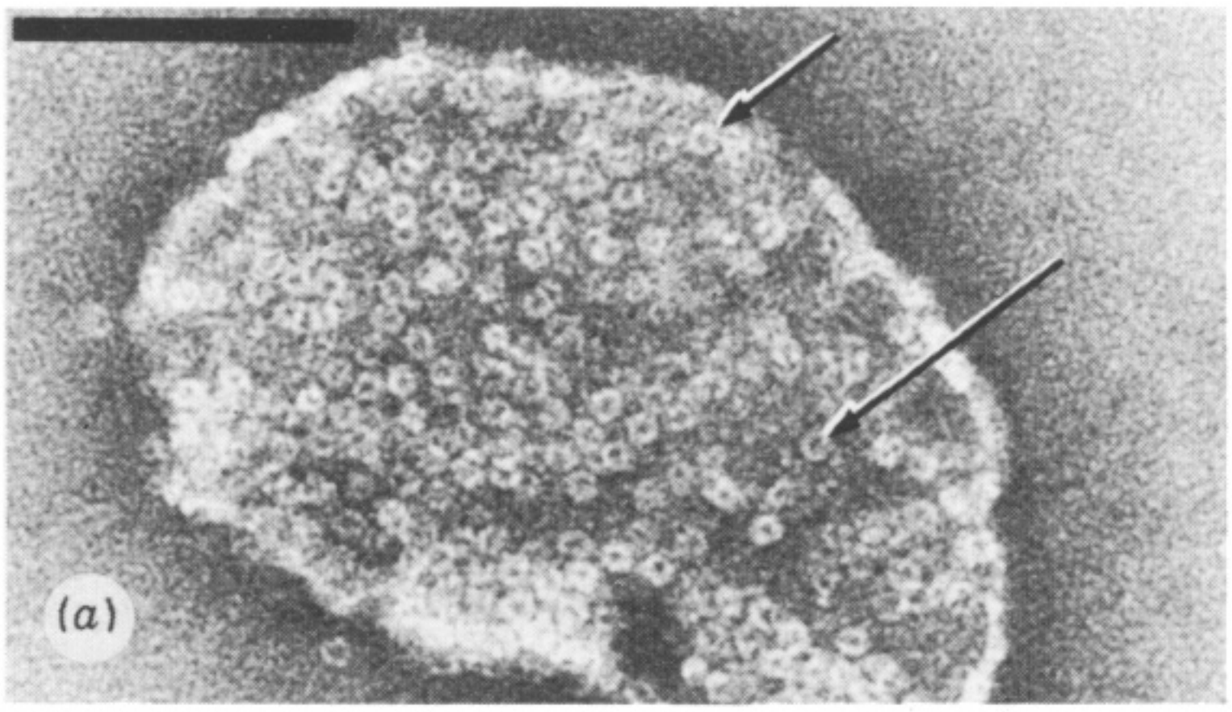
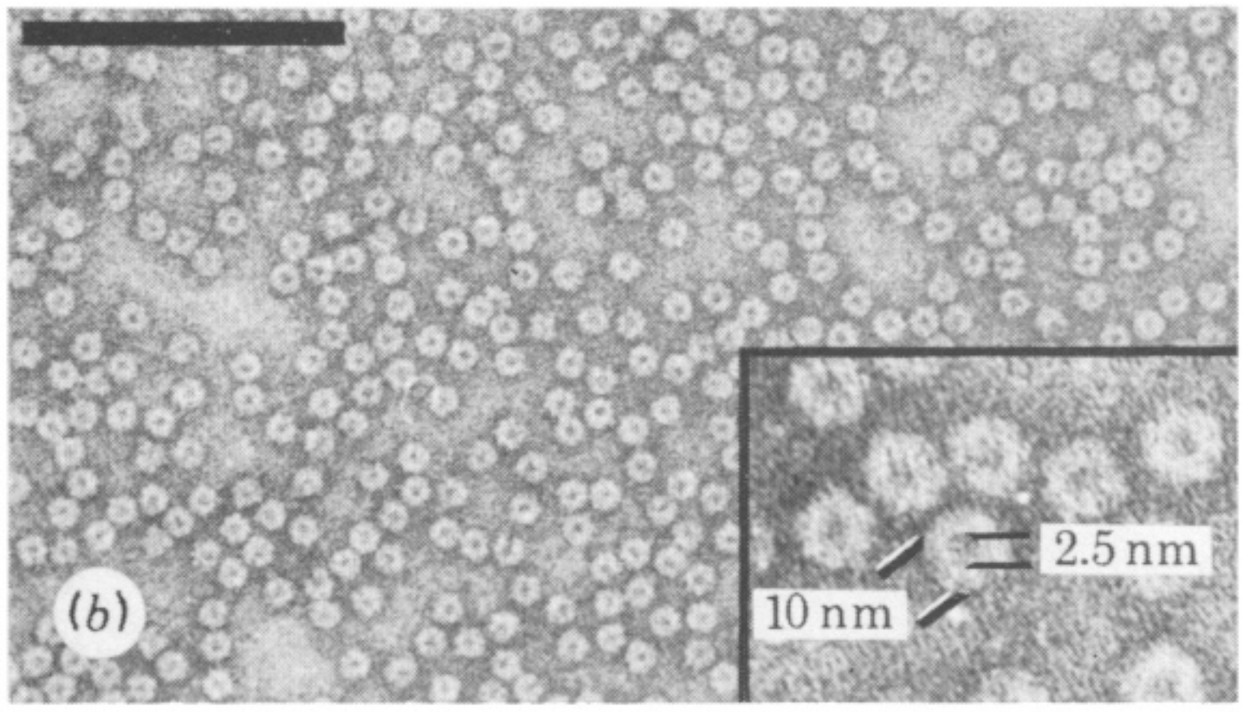
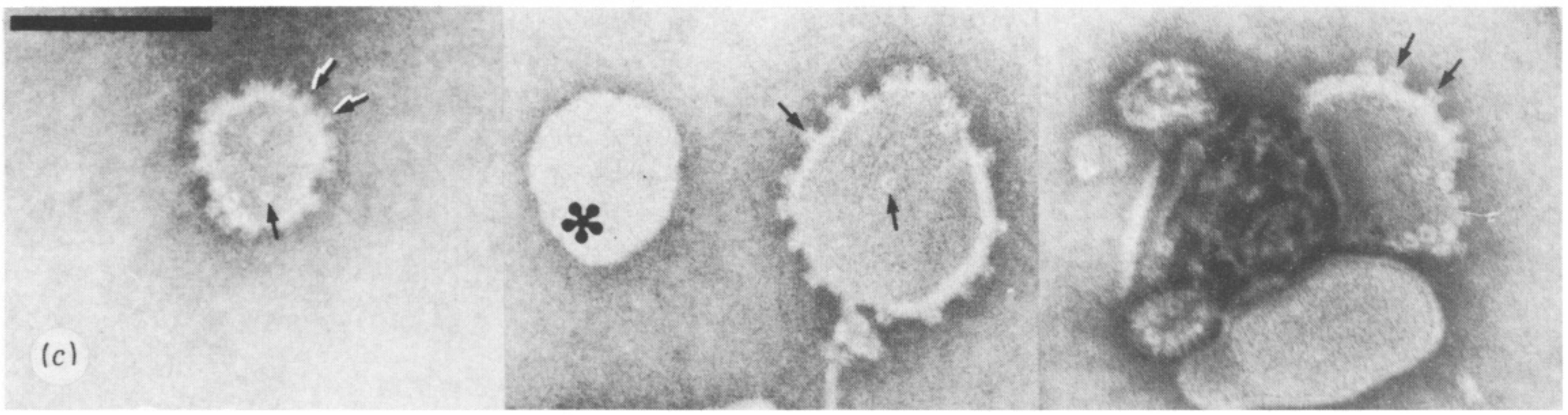
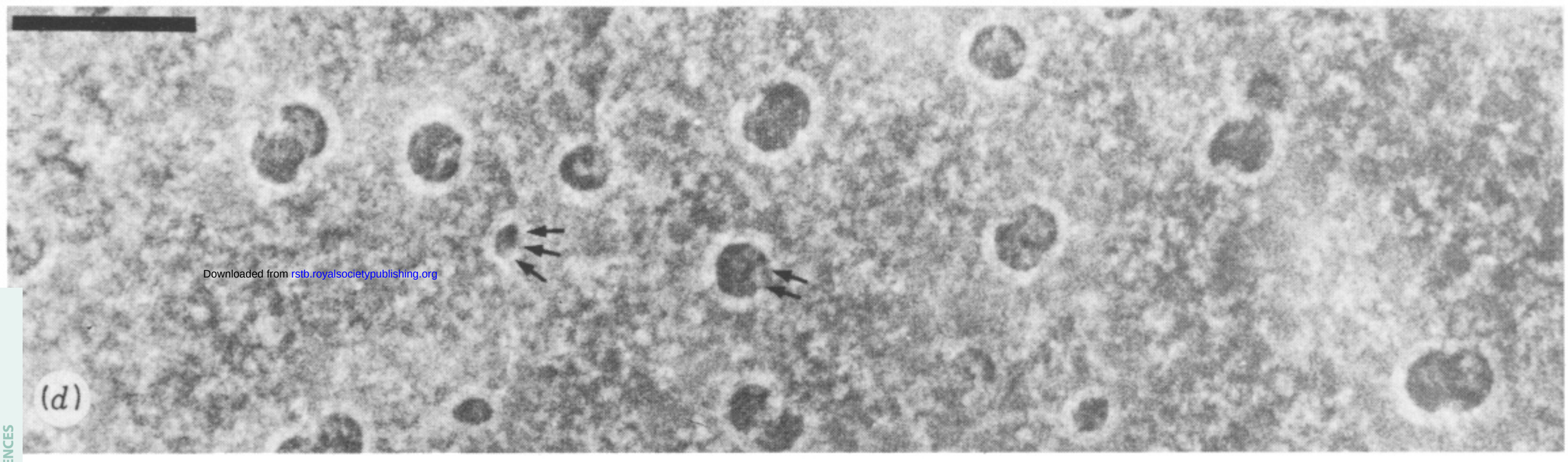


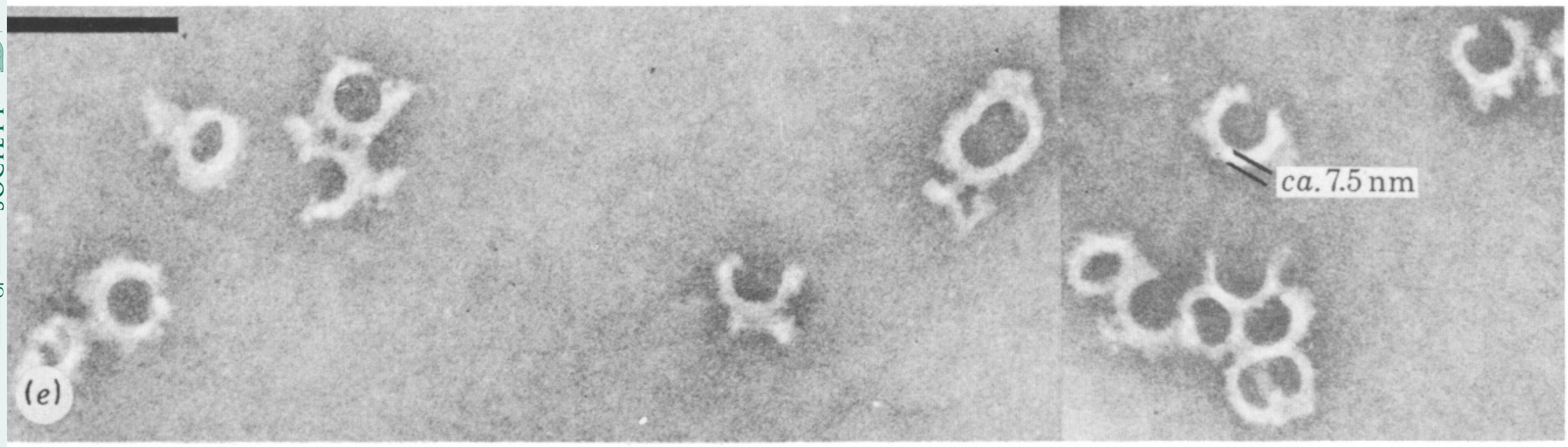
FIGURE 3. (*a*) and (*b*). Original demonstration of the presence of C5b-9 complexes on target erythrocyte membranes (from Bhakdi *et al.* 1975). Membranes were lysed with whole human serum, solubilized with Triton X-100 and analysed by double diffusion using anti-C5 (wells 1), anti-C6 (wells 2) and anti-C9 (wells 3, plate *b*). Fresh human serum was analysed in parallel as the control (plate *a*). Note the reaction of non-identity of native C5, C6 and C9 components (*a*), but the coalescence of the respective precipitates derived from membrane solubilisates (*b*) indicative of complex formation between these proteins, which had previously been identified as the membrane-damaging entities of the complement system (Lachmann & Thompson 1970; Thompson & Lachmann 1970). (*c*) Original demonstration that membrane-bound C5b-9(m) mimics intrinsic membrane proteins and is not eluted from intact membranes (from Bhakdi *et al.* 1975). Complement-lysed erythrocyte membranes were eluted by different salt solutions and the aqueous supernatants applied to wells 2 (elution with dilute EDTA), 5 (elution with 100 mM EDTA), and 8 (elution with 1.2 M NaCl). Membrane pellets before (wells 1, 4, 7) and after elution (wells 3, 6, 9, respectively) were applied in the same plate. Note the entire absence of C5b-9(m) in the salt supernatants as revealed by rocket immunoelectrophoresis with anti-C9. C5b-9(m) was quantitatively recovered in the pellet in every case (equal heights of rocket over wells 3, 6 and 9 compared to 1, 4 and 7, respectively).











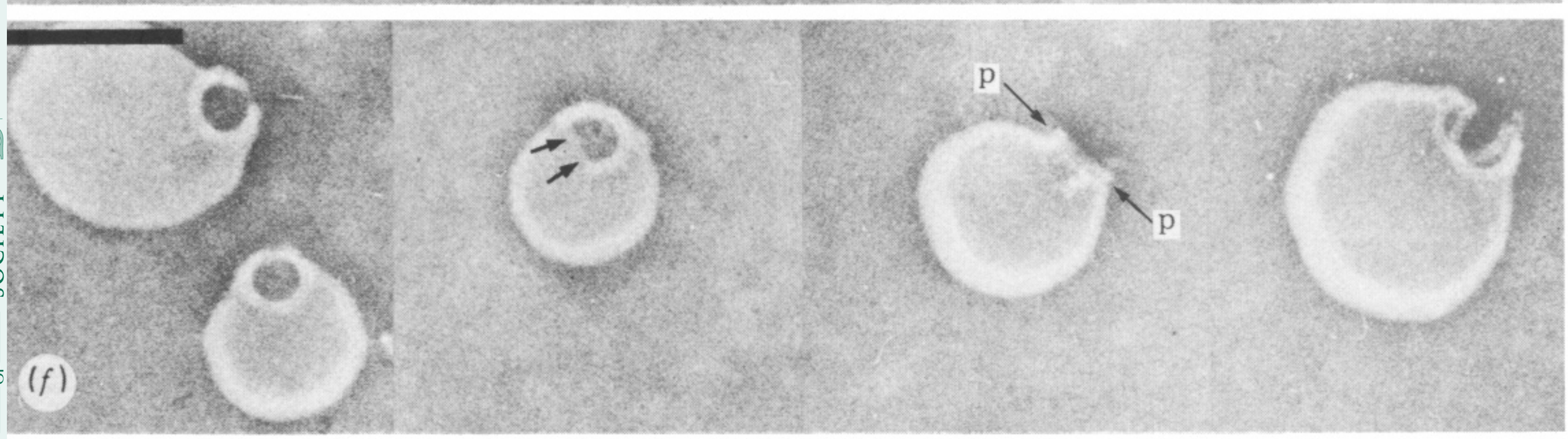
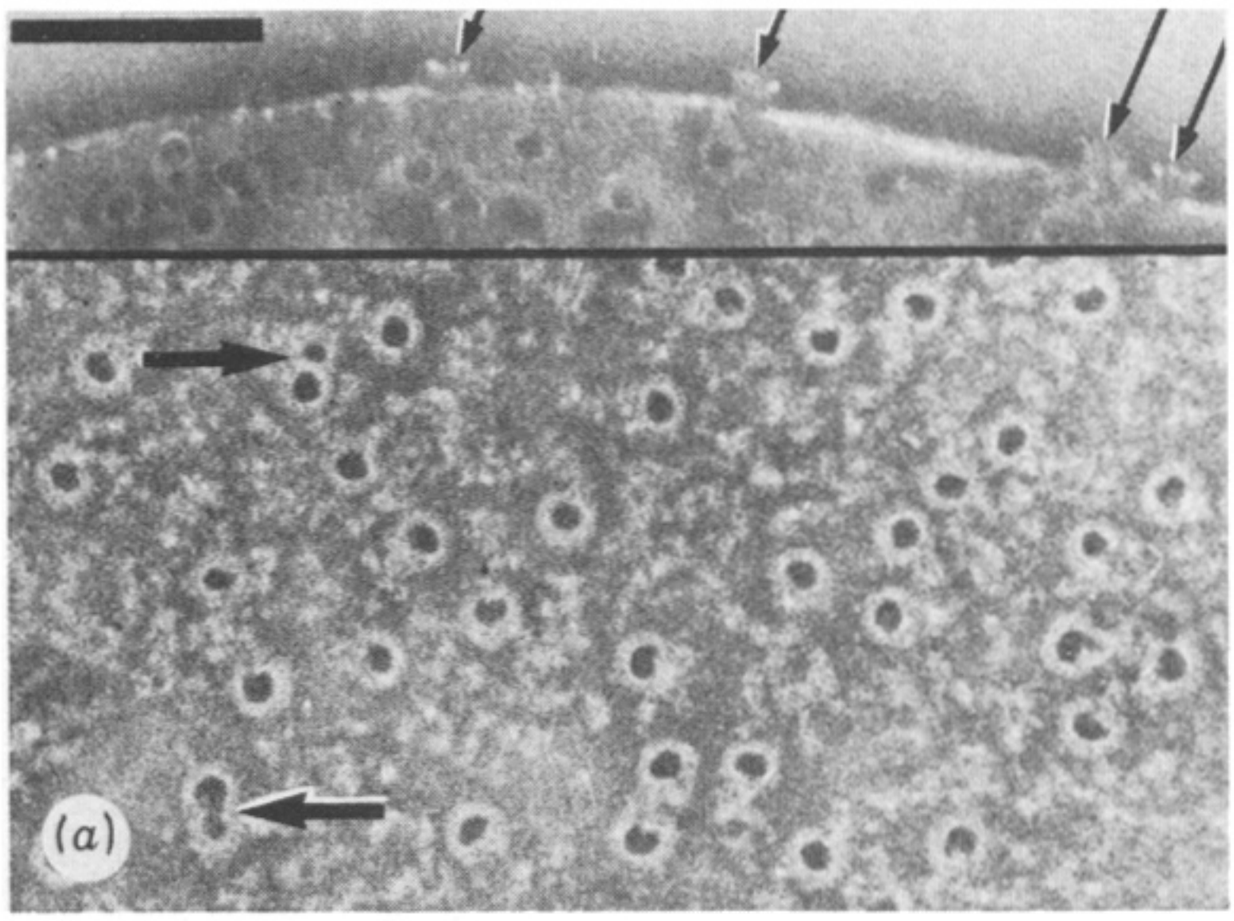
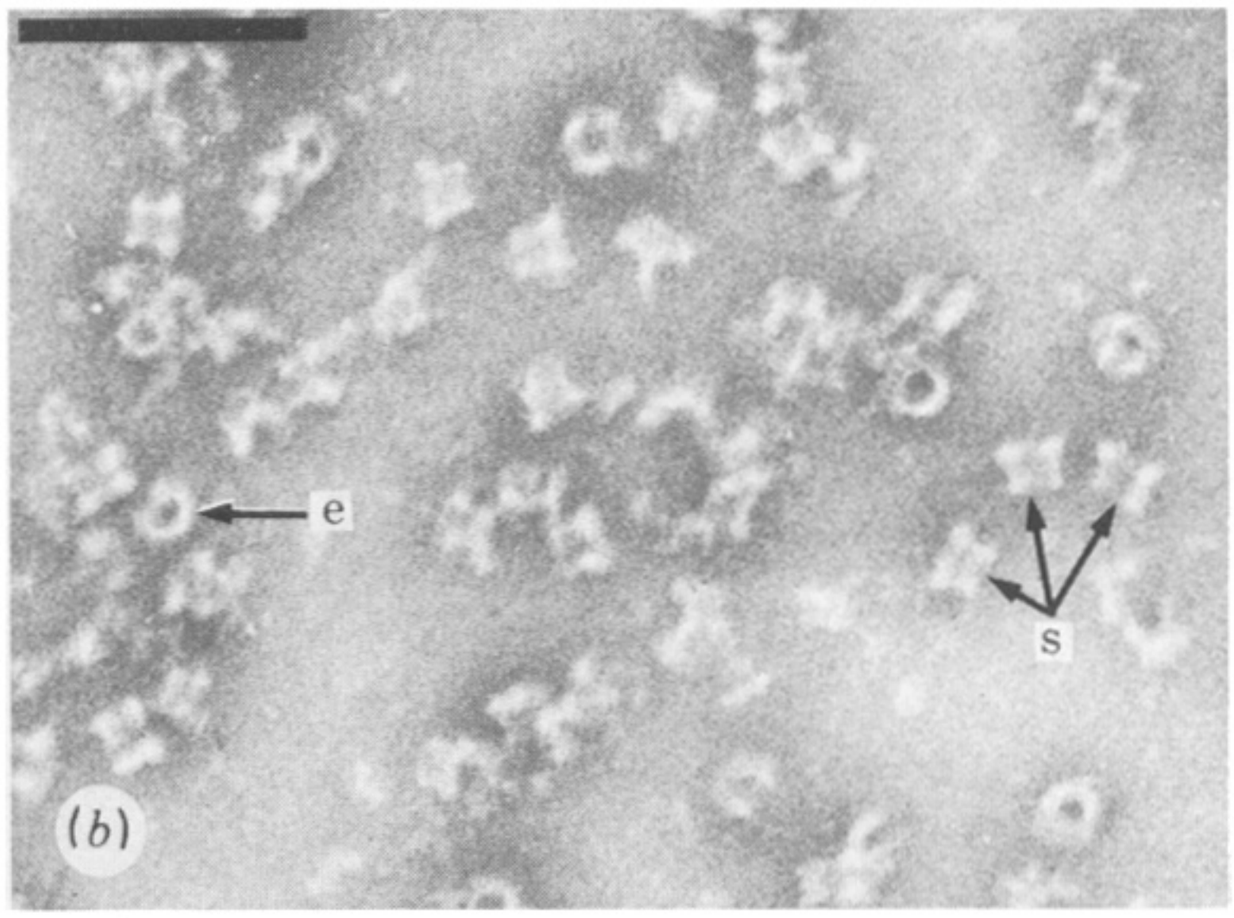
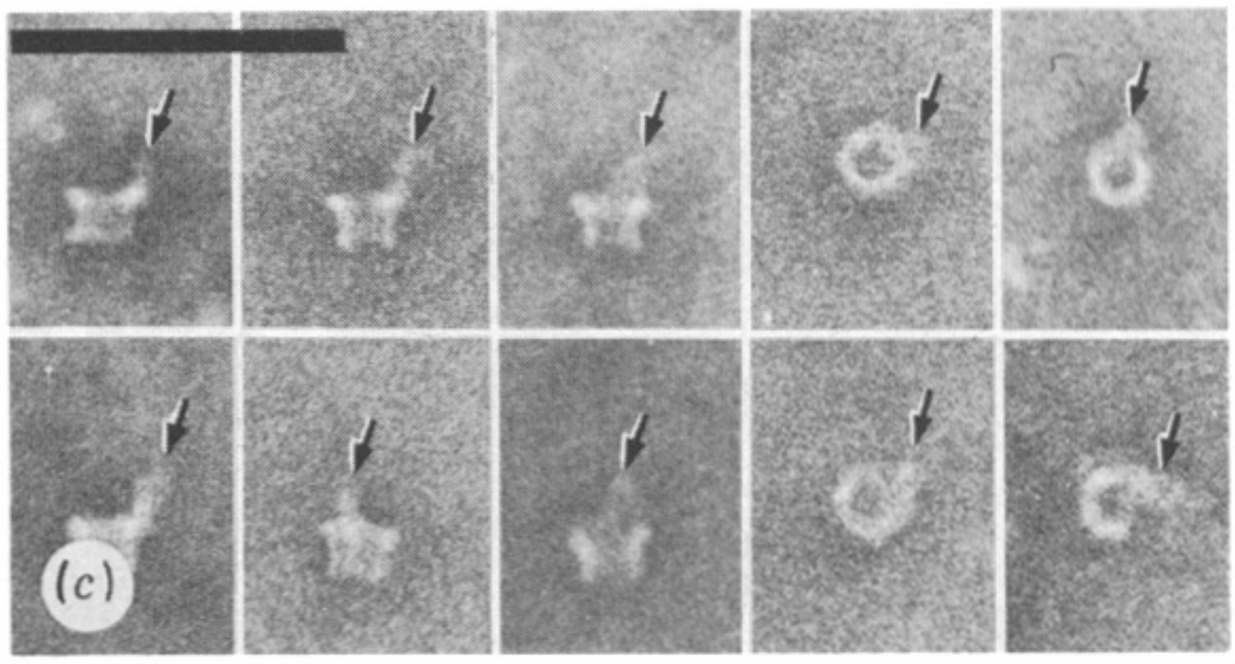
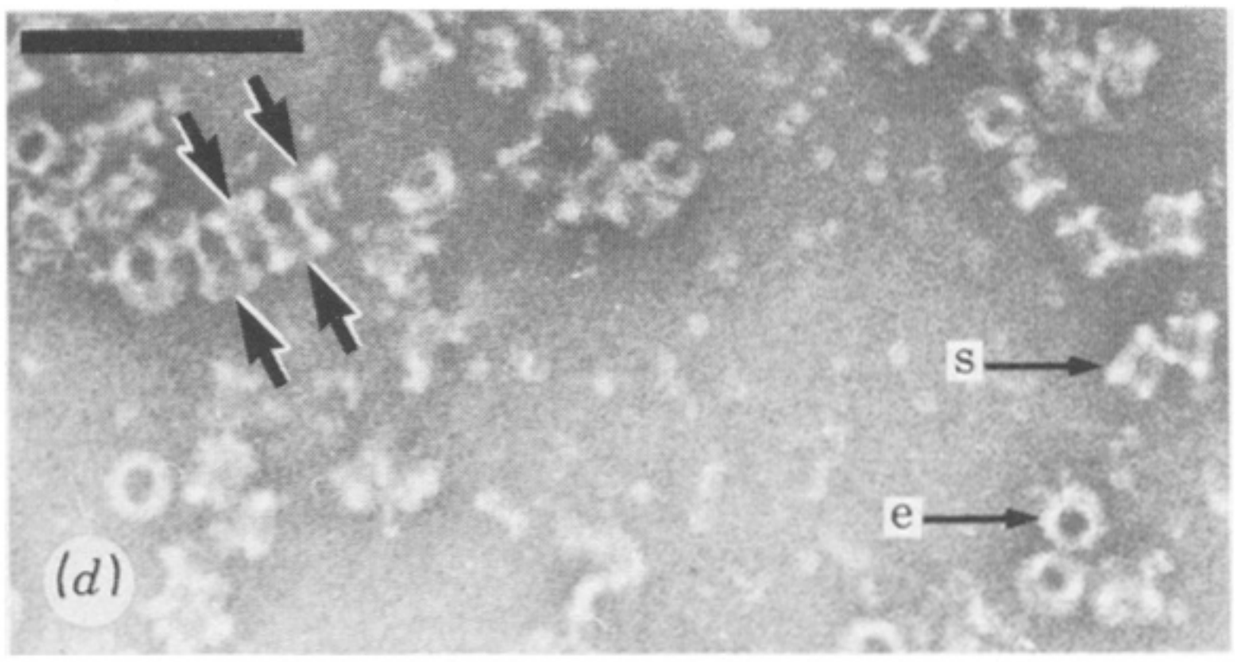


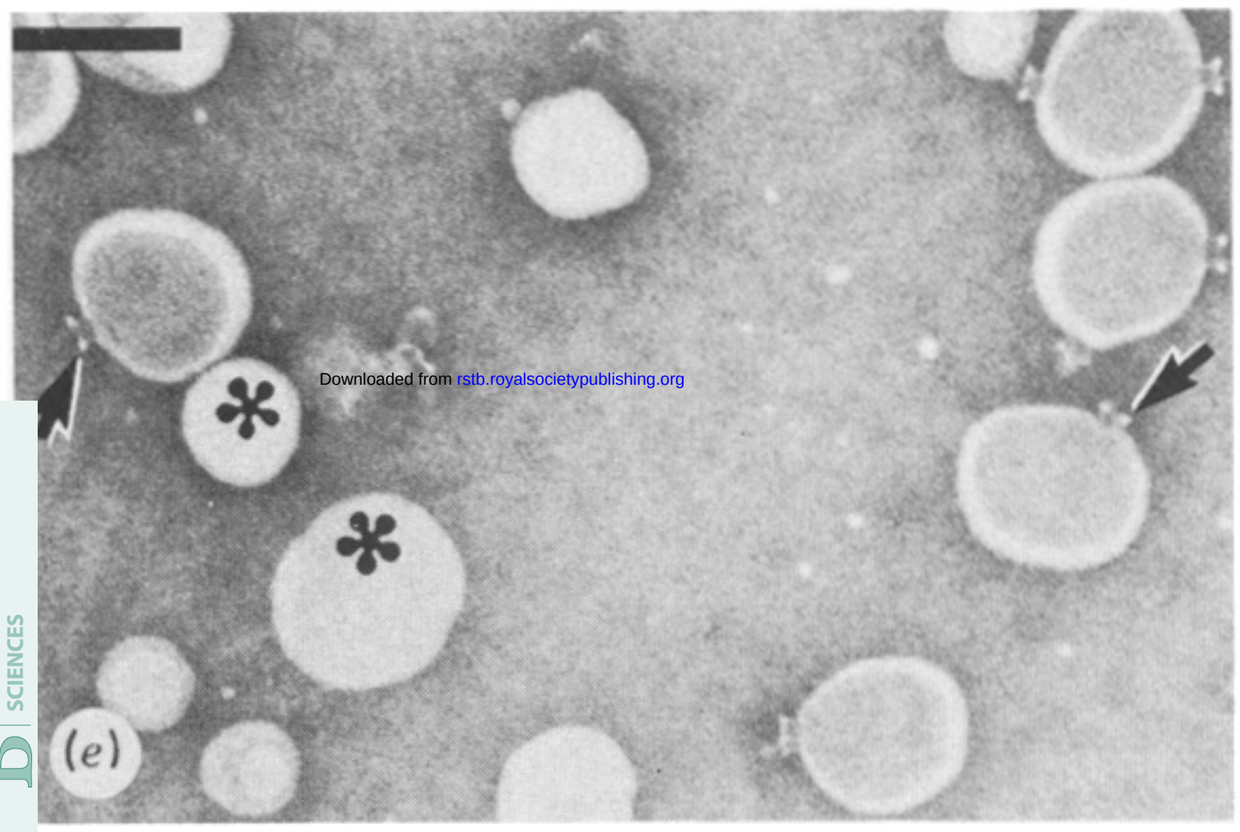
FIGURE 1. For description see opposite.

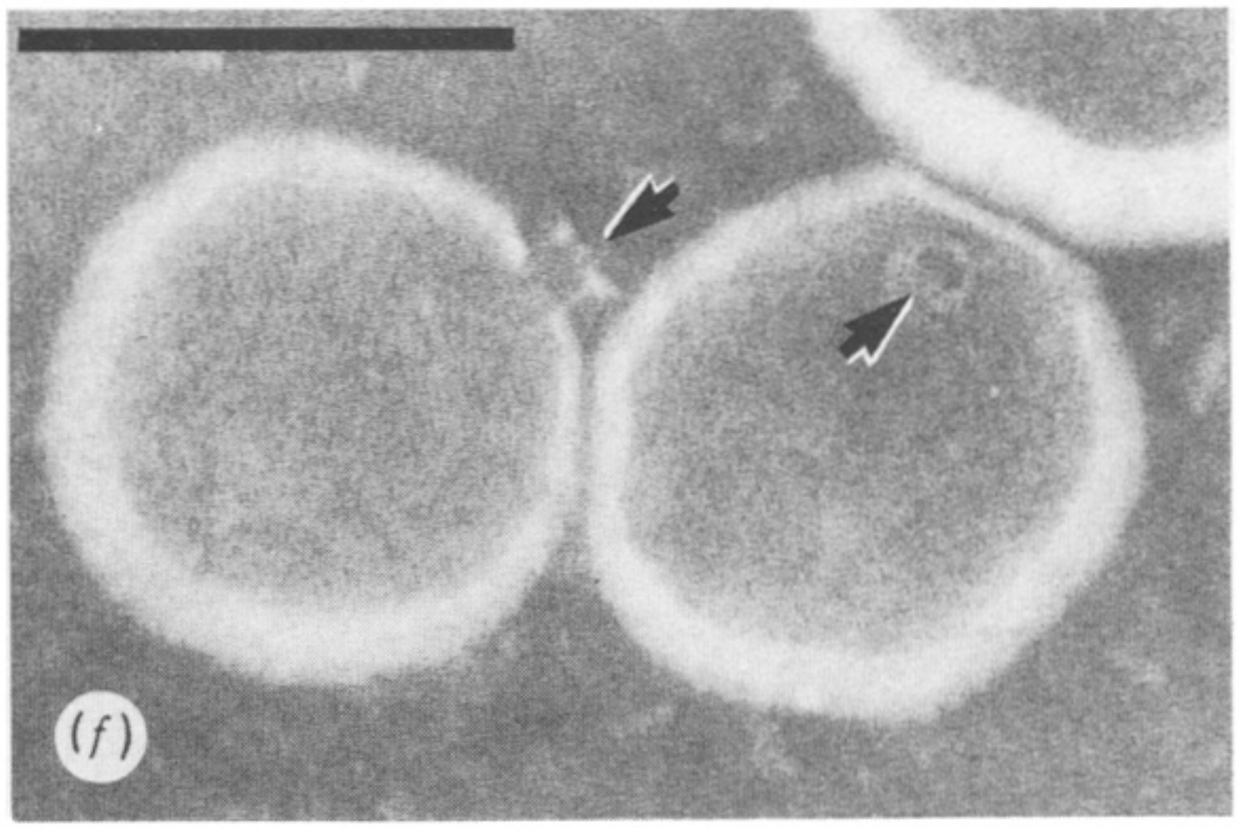












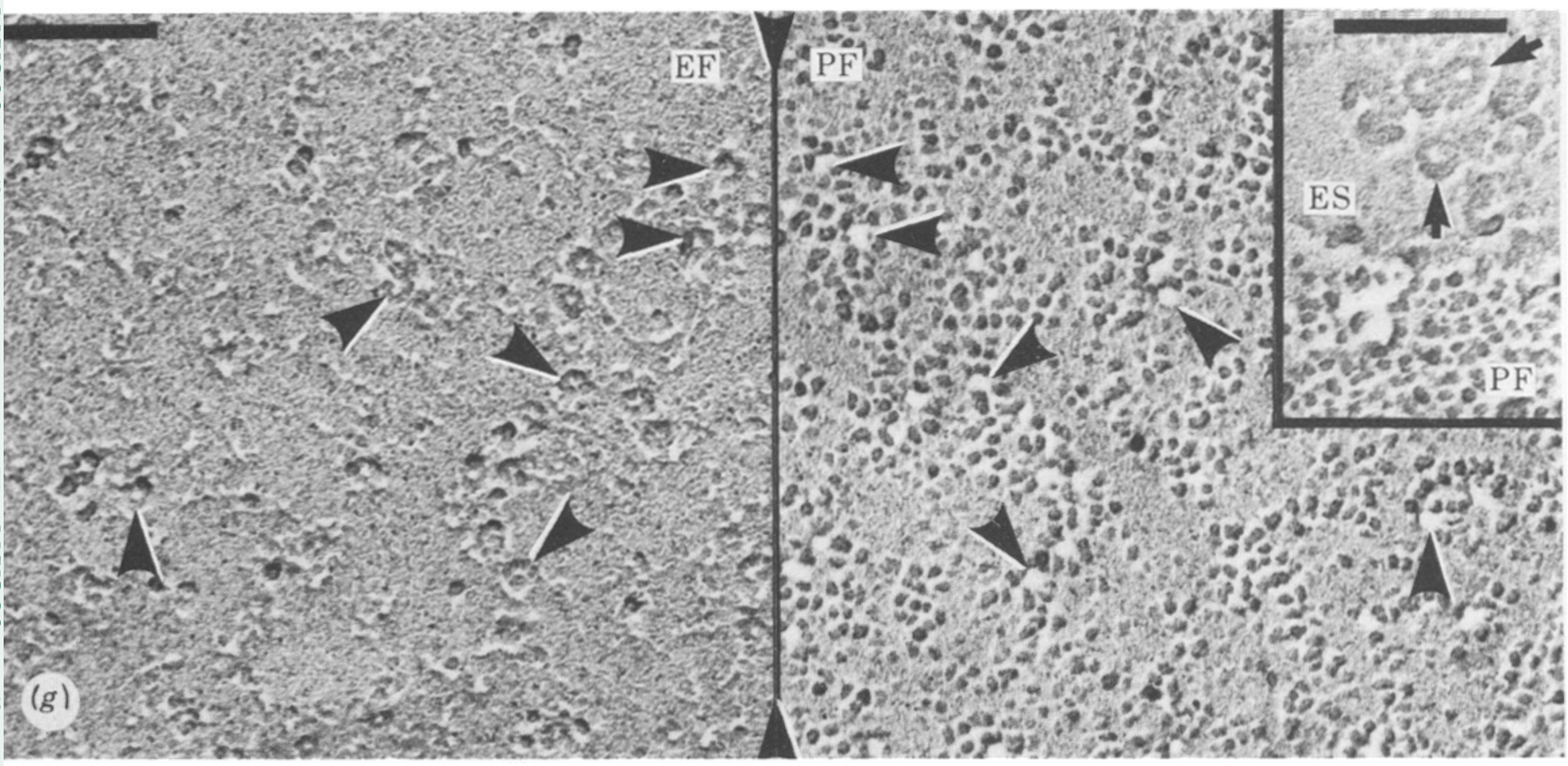
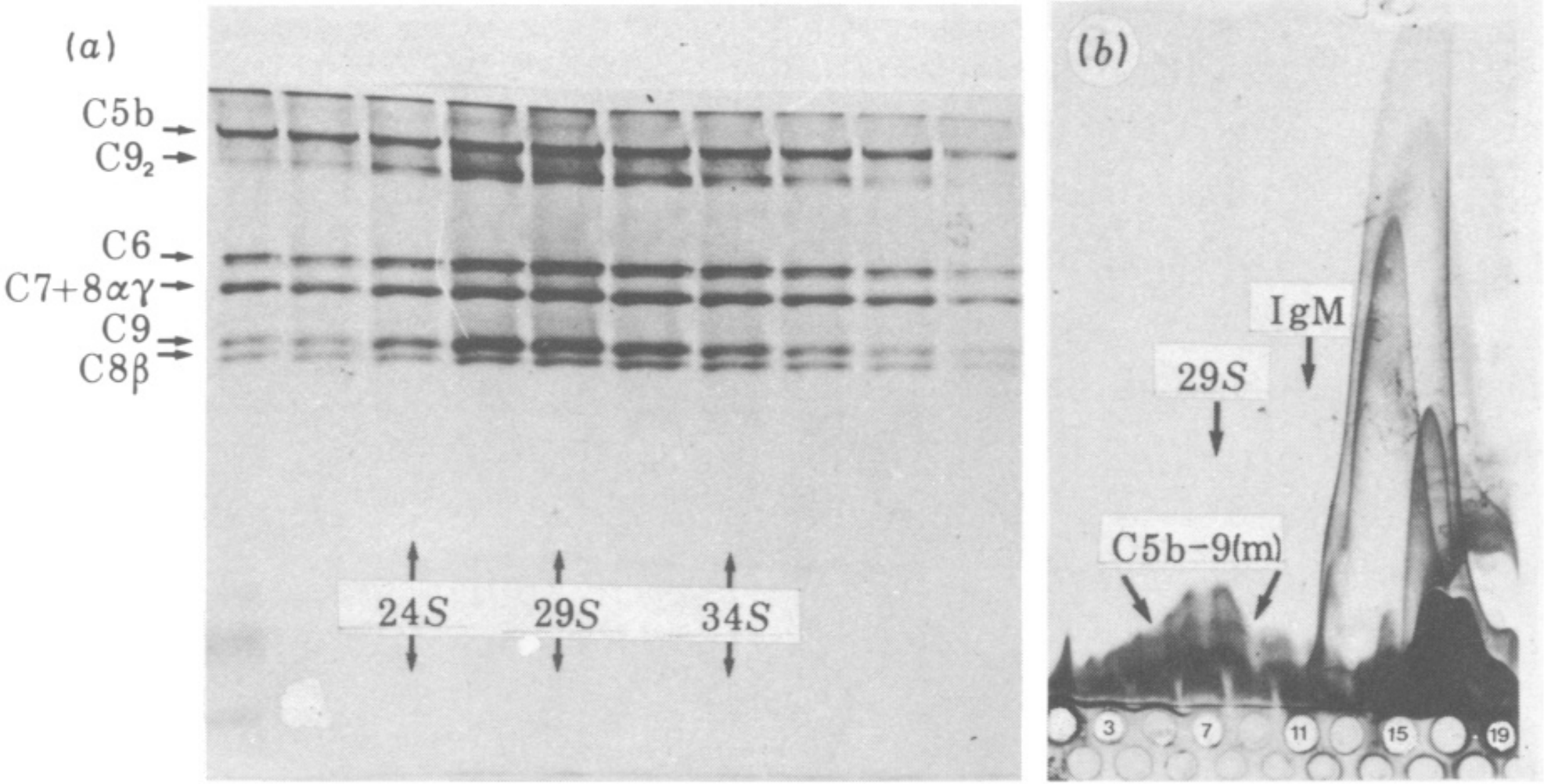


FIGURE 5. For description see opposite.



GURE 4. Isolation of C5b-9(m) from target erythrocyte membranes be sedimentation in detergent-containing sucrose density gradients. Washed membranes were solubilized with 250 mM deoxycholate and centrifuged in 10–43 % (by mass) sucrose density gradients containing 6.25 mM detergent. Twenty fractions were collected and analysed by SDS-polyacrylamide gel electrophoresis (plate *a*) and by fused rocket immunoelectrophoresis with the use of polyspecific antibodies to human serum proteins (plate *b*). Plate *a* shows the gel electrophoresis pattern obtained with the first 10 fractions from the gradient (corresponding to 25–40*S*). The C5b-9(m) complex exhibits a typical polypeptide composition and the individual subunits of the complex have been identified. Direction of sedimentation: right to left.