Biochimica et Biophysica Acta, 604 (1980) 1–26

BBA 85202

BACTERIAL CELL SURFACE AMPHIPHILES

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(Received August 31st, 1979)

Contents

I. Introduction

The term 'amphiphile' is used to describe a diverse range of polymers that share certain physico-chemical similarities, namely the presence of both hydrophilic and hydrophobic regions in their structure. Such amphiphiles or amphipathic molecules are present in all membranes but bacterial membranes contain specialized amphiphiles which have evoked considerable interest because of their serological and biological properties and their possible role in the pathogenesis of disease. The hydrophilic component of these amphiphiles is generally a charged polymer, as illustrated by the well-known lipopolysaccharides of Gram-negative bacteria and lipoteichoic acids of Gram-positive bacteria. More recent additions to this group are the enterobacterial common antigen in Gram-negative bacteria, lipomannans in *Micrococcus* sp. and a new and only partially characterized amphiphile from *Actinomyces* sp. The lipoproteins in Gram-negative enterobacteria also share a number of the properties of these amphiphiles.

In the case of Gram-negative bacteria the amphiphiles are detectable in the outer leaflet of the outer membrane and also to a lesser extent in the external environment. In the case of Gram-positive bacteria they are present in the cytoplasmic membrane but they can generally be detected as surface components and often to quite a significant extent as extracellular components.

The purpose of this article is to summarize and compare the known classes of these amphiphiles with respect to their structure, locational mobility, physicochemical and biological properties as well as their potential and known role in pathogenesis of disease.

II. Chemistry

Fig. 1 summarizes schematically the known structure of the various types of classes of amphiphile and stresses on the one hand their chemical diversity and on the other their common features of amphipathicity (hydrophilic and hydrophobic regions), large molecular size and charge.

IIA. Lipopolysaccharides

Lipopolysaccharides are the oldest known example of amphiphile and have been extensively studied for well over a century both in terms of their chemistry and biological properties (for reviews see Refs. 1–6).

Much of our knowledge of the structure of lipopolysaccharides has come from studies on *Salmonella* sp. As illustrated in Fig. 1, the molecule consists of three distinct regions covalently linked together, namely the hydrophilic polysaccharide and core regions and the hydrophobic lipid component. The polysaccharide is composed of repeating oligosaccharide units, which often contain rare sugars and which, in the case of *Salmonella*, enable species to be identified by their *O*-specific antigens. Studies with a variety of genera indicate that there is considerable diversity in the structure of the polysaccharide component, and that the presence of immunodominant carbohydrate components provides the basis for a serological classification. The core region, on the other hand, is generally regarded as being of constant composition for smooth strains from a particular genus although differences occur in the core structure of different genera. Two components which are regarded as characteristic are aldoheptose and 2-keto-3-deoxyoctonate although there are exceptions [7]. Ketodeoxyoctonate, apart from contributing along

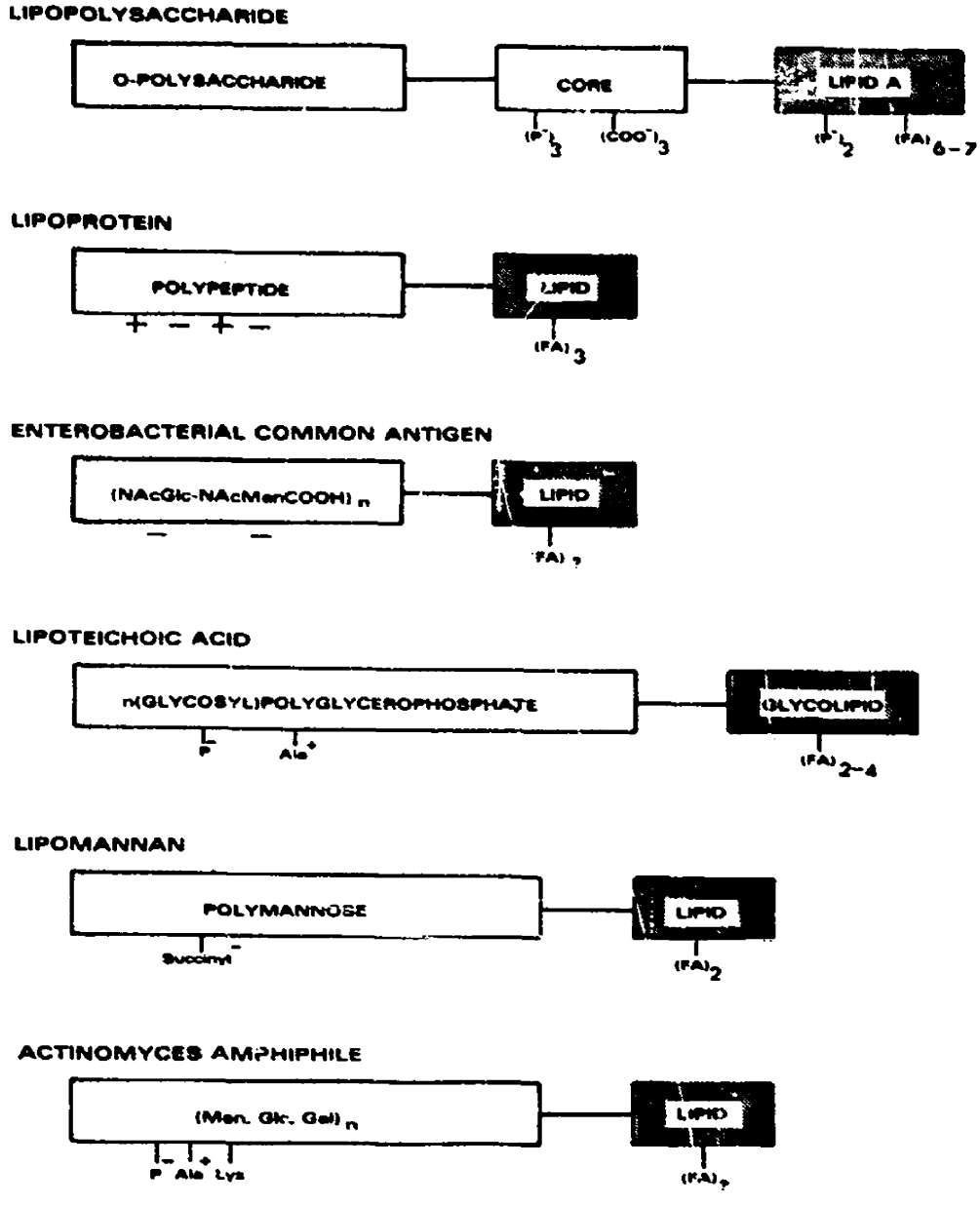

Fig. 1. Schematic representation of known and proposed structures of various classes of bacterial amphiphile. The figures stress the essential amphipathic character of these molecules and indicate the nature of charged substituents on the hydrophilic portion of the polymers. Terminal positioning of the hydrophobic or lipid moiety in the cases of the enterobacterial common antigen and the *Actinomyces* amphiphile is speculative and based on analogy with the known structure and properties of other amphiphiles. Symbols: P^-, phosphate; COO^-, carboxyl; FA, fatty acid ester; Ala, alanine; Lys, lysine; Glc, glucose; Gal, galactose, Man, mannose; NAcGlc, *N*-acetyl-D-glucosamine; NAcManCOOH, *N*-acetyl-D-mannuronic acid.

with phosphate to the net negative charge, provides the linkage, through an acid-labile bond, to the lipid A region of the molecule.

The lipid A regions of lipopolysaccharides from a wide range of Gram-negative bacteria also share a general structural similarity in which there are phosphorylated glucosamine residues forming a disaccharide to which are attached fatty acids by ester and amine bonds; characteristically the *N*-acyl residue is a 3-hydroxy alkanoic acid, such as 3-hydroxytetradecanoic (myristic) acid. Ethanolamine or other amino compounds (e.g. 4-aminoarabinose) are also often present.

Microheterogeneity can exist in all regions of the molecule such as the existence of side chains differing in length and substitution in the same lipopolysaccharide preparation. In rough mutants the side chains are missing and may also be absent from some core regions of lipopolysaccharide isolated from wild type bacteria. Indeed it can be said that lipopolysaccharide preparations are not homogeneous [5].

IIB. Enterobacterial common antigen

Enterobacterial common antigen is an antigen shared by almost all wild-type strains of Enterobacteriaceae [8–11]. It has been isolated from *Salmonella montevideo* and shown to be a linear polymer of 1,4-linked *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosaminuronic acid esterified to a small extent by palmitic and acetic acids. While 70% of the polymer can be accounted for by those components, the low water solubility of some preparations is perhaps suggestive of further, at present unknown, lipid substitution [10, 11]. A further complication is its occurrence in two different forms, one a haptenic or free form of 2700 molecular weight and the other an immunogenic form which is restricted to a few R-form bacterial strains where it is covalently linked to the core region of the lipopolysaccharide [11]. While chemical characterization of enterobacterial common antigen is incomplete it can clearly be regarded as an amphiphile from its amphipathic and charged character.

IIC. Lipoproteins

Lipoprotein was first isolated and characterized from *Escherichia coli* [12,13] and similar structures have since been shown to occur in a wide range of Gram-negative bacteria. It is the most abundant protein in the outer cell membrane of *E. coli* and contains 58 amino acids in a predominantly helical polypeptide. The N-terminal end is a cysteine residue which is rendered hydrophobic by substitution of the α-amino group with a fatty acid and the mercapto group with a diglyceride; approximately one third of the C-terminal residues are in covalent linkage to the peptidoglycan layer of the cell envelope, the rest being present in a free form.

IID. Lipoteichoic acids

Lipoteichoic acid was first isolated from *Lactobacillus fermentum* [14] and similar structures have subsequently been shown to be present in a wide range, but not all, of Gram-positive bacteria [15–18]. They can be distinguished from the earlier known cell wall teichoic acids on the basis of their structure and lack of covalent association with wall peptidoglycan. Lipoteichoic acids are typically linear polymers of glycerophosphate some 25–30 residues long linked 1–3 by phosphodiester bonds. The 2-position of the

glycerol residues can be substituted variously with sugars in glycoside linkage and D-alanine in ester linkage. The phosphomonoester end of the polymer is covalently linked to a lipid. The latter may take the form of a glycolipid as in the lactobacilli and some streptococci [14,16,17,19], a phosphatidylglycolipid as in *Streptococcus faecalis* [20,21] or simple fatty acid substitution of the terminal glycerol moiety as has been reported for a mutant of *Bacillus licheniformis* that lacked the ability to form glycolipid [22]. The lipid moiety is generally also found as a free lipid constituent of the plasma membrane. Fatty acid substitution generally reflects the overall fatty acid composition of the membrane lipids of a particular species and in this sense lipoteichoic acid preparations are inevitably heterogeneous. In species where lipoteichoic acids normally occur, their presence is much less subject to cultural conditions than the cell wall teichoic acids and, while this might denote an importance of lipoteichoic acids in the economy of the producing cell, it probably reflects a biosynthetic route for lipoteichoic acids that is completely separate from that for wall teichoic acids [21,23,24].

IIE. Lipomannans

As indicated above not all Gram-positive bacteria contain lipoteichoic acid. The genus *Micrococcus* has instead a lipomannan, a linear polymer of some 52–70 D-mannose residues about a quarter of which are succinylated. A glycolipid covalently joined to one end of the polymer provides the hydrophobic region of the molecule [25,26].

IIF. Other amphiphiles

Actinomyces sp. are similarly known to lack lipoteichoic acids. Recently a complex and as yet incompletely characterized amphiphile has been isolated from *A. viscosus* and also been detected serologically in other species of actinomycetes [27]. It appears to be a fatty-acid substituted heteropolysaccharide of mannose, glucose and galactose. Other substituents, which would contribute to the overall net negative charge of the molecule, are glycerophosphate, lysine and alanine. The F or Forssman antigen of *Diplococcus pneumoniae* has amphipathic properties [28] and while its structure is unknown it probably takes the place of lipoteichoic acid in this organism; both types of amphiphile have been implicated as inhibitors of cell wall autolysins (see Section VIC-3). Strains of *Streptococcus mitis* have also recently been reported to lack lipoteichoic acids [29] but it is not known whether these organisms possess other amphipathic species.

III. Isolation and criteria of purity

IIIA. Lipopolysaccharides

A variety of mild extraction procedures have been used to obtain lipopolysaccharides in a water-soluble form. These include 45% aqueous phenol, 65–68°C [30]; aqueous phenol/chloroform/light petroleum 5–20°C for R-form bacteria [31]; 0.25 M trichloroacetic acid, 4°C [32]; aqueous EDTA (pH 8.0–8.5), 37°C [33]; aqueous butan-1-ol, 0–4°C [33]; dimethylsulphoxide, 60°C [34]; 1 M NaCl/0.1 M sodium citrate (pH 7.0), 0–4°C [35] and aqueous diethyl ether at ambient temperature [36].

The most frequently employed method is hot aqueous phenol extraction of whole organisms, although cell disruption, either mechanically or by lysozyme treatment in the

presence of EDTA, has been reported to increase the yield of lipopolysaccharide on subsequent aqueous phenol extraction [37]. The enterobacterial common antigen has also been obtained by similar procedures [8,9]. To obtain the lipopolysaccharide in the hot aqueous phenol extract the mixture is cooled and centrifuged to separate the phases. The lipopolysaccharide is usually present in the aqueous phase though in a few instances, such as *Leptotrichia* [38], lipopolysaccharide is present in the phenol phase. The observation that the more hydrophobic lipopolysaccharides of R-form mutants and *Veillonella* [39] are poorly extracted by aqueous phenol is perhaps indicative of an important role for hydrophobic interactions. Such lipopolysaccharides are extracted in higher yields and high purity by the phenol/chloroform/light petroleum method. The composition of the extract from a particular organism is influenced by the extraction procedure and further purification is generally necessary to remove contaminants which may include nucleic acids, polysaccharides, membrane lipids and proteins. Proteins in particular bind tenaciously to lipopolysaccharides and for whole cell extraction the hot aqueous phenol method is the best in terms of yielding a product low in protein.

IIIB. Lipoteichoic acids

Most of the extraction procedures used for lipopolysaccharide have been compared [40] in the extraction of lipoteichoic acids. Trichloroacetic acid is precluded in that it results in loss of the lipid portion of the molecule, while 45% aqueous phenol gives the largest yield of the apparently undegraded molecule. Variations of the aqueous phenol extraction procedures have been used to obtain lipoteichoic acids from a variety of Gram-positive bacteria [40,41] and indeed the method has been extended to the extraction of other amphiphilic structures from organisms where lipoteichoic acids are absent, such as *Micrococcus* and *Actinomyces* sp. [25,26,27].

With respect to lipoteichoic acids, extraction with 45% aqueous phenol at 65–68°C gives a product low in contaminating protein while extraction at lower temperature gives higher contaminating protein but better preservation of D-alanyl substituents [41]. In both procedures the long-chain fatty acid ester residues of the lipid moiety of lipoteichoic acids are retained. For complete extraction of lipoteichoic acid from small quantities of cells (milligram quantities) the addition of 0.01 M Mg^{2+} to the aqueous phase of the extraction milieu has been shown to be very effective (see Ref. 78) in certain instances.

IIIC. Purification procedures

While 45% aqueous phenol appears to be useful in extraction of most classes of amphiphile (with the possible exception of lipoprotein for which boiling sodium dodecyl sulphate is the preferred procedure [12]), a variety of conditions should be examined with any new organisms or amphiphile studied with respect to final yield and purity of product. Whatever method is used the extract will contain varying amounts of contaminating polynucleotides, proteins and polysaccharides and, with some extraction procedures, lipids.

Further purification usually involves ultracentrifugation in the case of lipopolysaccharides or gel-permeation chromatography in the case of the other classes of amphiphile [1,2,3,16,17,20,26,27]. Digestion with DNase and RNase prior to such second stage purification can largely eliminate polynucleotide contamination and organic solvent extraction is useful in removal of unbound lipid where the amphiphile is insoluble in the solvent. The other major sources of contamination, protein and polysaccharide, present

much more difficult problems. The tendency of amphiphiles to form tenacious complexes with these macromolecules demands caution in determining the purity of preparations. Protein can largely be eliminated from lipopolysaccharides by hot aqueous phenol as has already been mentioned, but the detection of polysaccharide contaminants in such amphiphiles which are themselves complex heteropolysaccharides becomes much more difficult. The situation is complicated further when such contaminants can also be constituents of lipopolysaccharides, viz. the enterobacterial common antigen in its immunogenic form. Recently Galanos and co-workers [42] have achieved an extremely high standard of purification and homogeneity for *Salmonella abortus equi* lipopolysaccharide. Protein was entirely eliminated from the preparation by applying the phenol/chloroform/light petroleum extraction procedure to phenol/hot water extracts of the organism. Further purification by ultracentrifugation and electrodialysis to a uniform salt form has resulted in a standardised lipopolysaccharide preparation (Novo-Pyrexal) which is commercially available.

Polysaccharide and protein contamination of lipoteichoic acid preparations while more readily recognisable chemically is not resolved by the simple gel-permeation chromatography used for partial purification of phenol/water extracts [16–18]. Treatment with broad spectrum proteases will lower but not entirely eliminate protein. Lectin and hydrophobic affinity chromatography has been used with limited success in further purification of lipoteichoic acids (Ref. 43 and unpublished observations). Recently the novel technique of the incorporation of lipoteichoic acid into liposomes and subsequent retrieval by organic solvent extraction has been reported to remove contaminating polysaccharide [44], albeit the method is only applicable to the purification of milligram quantities of lipoteichoic acid. Ion-exchange chromatography also suffers from extreme limitations when used for amphiphile purification. A combination of hydrophobic and ionic interaction between amphiphile and ion-exchange resin generally makes for low and variable recoveries from such matrices [17,18] even in the presence of high salt concentrations and the eluted material may reflect fractions of relatively low hydrophobicity that are not representative of the whole preparation. The incorporation of detergents such as sodium dodecyl sulphate or Triton X-100 does allow for quantitative or near quantitative recovery of amphiphiles from ion-exchange systems and provides useful purification tools when chemical or structural work on the amphiphile is the immediate goal. If, however, the purpose of purification is to study biological properties that involve membrane interaction, complete and certain removal of the detergent becomes a major problem. What are clearly needed are methods for the complete purification of amphiphiles that do not risk contamination with membrane-active chemicals and also afford yields of amphiphile that can be considered representative of the starting material. Until this is achieved some doubt must be exercised as to the real significance of some of the plethora of biological activities that have been reported in the literature (vide infra).

While purity of amphiphile preparations can be defined in terms of the presence or absence of expected contaminants heterogeneity is 'built-in' into many amphiphiles. This is particularly striking in the case of lipopolysaccharides where there is clear evidence of different molecular species in individual preparations of lipopolysaccharide. For example, physically separable fractions of lipopolysaccharide, differing greatly in the lengths of their side-chains, are present in *E. coli* 0111:B4 [45], and differences in composition and degree of polymerization of the side-chain have been found in other strains of *E. coli* and other genera [46–48]. The degree of heterogeneity in other amphiphiles, apart from fatty acid substituents, remains to be determined.

IV. Physico-chemical properties

Amphiphiles in aqueous solution tend to form micellar aggregates of one form or another to occlude water from the hydrophobic regions of their molecular structure. While the forces involved in this self-aggregation are probably largely hydrophobic, metal cation ionic bonding and hydrogen bonding could also be involved. Solutions of lipopolysaccharides are generally opalescent and both sedimentation studies and electron microscopy reveal a polydispersity of aggregates with particle weights of several million daltons. The shape of the individual particles varies through ribbons, discs and vesicles depending on both the organism of origin and the isolation procedure. These micellar aggregates can be dispersed by surfactants or chelating agents such as EDTA. The solubility of lipopolysaccharides can be altered by conversion to uniform cationic salt forms by electrodialysis [49,50] and this in turn can lead to changes in biological properties (vide infra). The existence of other amphiphiles in aqueous solution as micellar aggregates is assumed both from their chemical structure and behaviour on gel-permeation chromatography following treatment with detergents or chemical deacylation. Lipoteichoic acids, for instance, have a K_{av} of around 0.1 on columns of 6% agarose gels but following deacylation or treatment with detergents the K_{av} is increased to 0.5 [14,17]. Lipoteichoic acid, from which the fatty acid residues have been removed by mild chemical or enzyme hydrolysis, behaves similarly to detergent-treated lipoteichoic acid on agarose gels, the inference being that in the absence of hydrophobic interaction the lipoteichoic acids behave as monomers. Convincing electron micrographs of lipoteichoic acid micelles have yet to be obtained but it would appear that the micellar structure of lipoteichoic acid is smaller and less bizarre than that of lipopolysaccharide (Wicken, A.J., unpublished observations). Unlike lipopolysaccharides, where metal cations and polyamines are associated with 'native' material, lipoteichoic acid preparations as examined by Energy Dispersive X-ray analysis are not associated significantly with Ca^{2+} or Mg^{2+} (MacKay, M.A. and Wicken, A.J., unpublished observations) but will readily bind cations if these are added to lipoteichoic acid solutions [51]. Electrodialysis of lipoteichoic acid preparations in an attempt to alter micellar size by changing the nature of associated cations did not produce a demonstrable effect and it is suggested that the small amount of protein that remains tenaciously in such preparations acts as a counter-cation. Similarly other amphiphiles have been reported to form micellar aggregates in aqueous solution, which is not surprising in view of the chemical structure of this group of compounds [12,25,27].

V. Location of amphiphiles in the producing cells

VA. Gram-negative bacteria

Gram-negative bacteria have typically a cell envelope of three layers, namely an outer membrane, a layer of peptidoglycan and an inner cytoplasmic membrane. The three classes of amphiphile thus far isolated from Gram-negative bacteria are detectable in the outer membrane (Fig. 2). In *Salmonella typhimurium*, the outer membrane is made up of lipopolysaccharide, protein (including lipoprotein) and phospholipids in a weight ratio of 0.3 : 1.1 : 0.3 [52].

The use of ferritin-labelled antibodies [53] showed that lipopolysaccharide was in the outer membrane and projected some 300 Å beyond it. Preparations of outer membrane made at 0°C show only lipopolysaccharide on the outer face, at high temperatures trans-

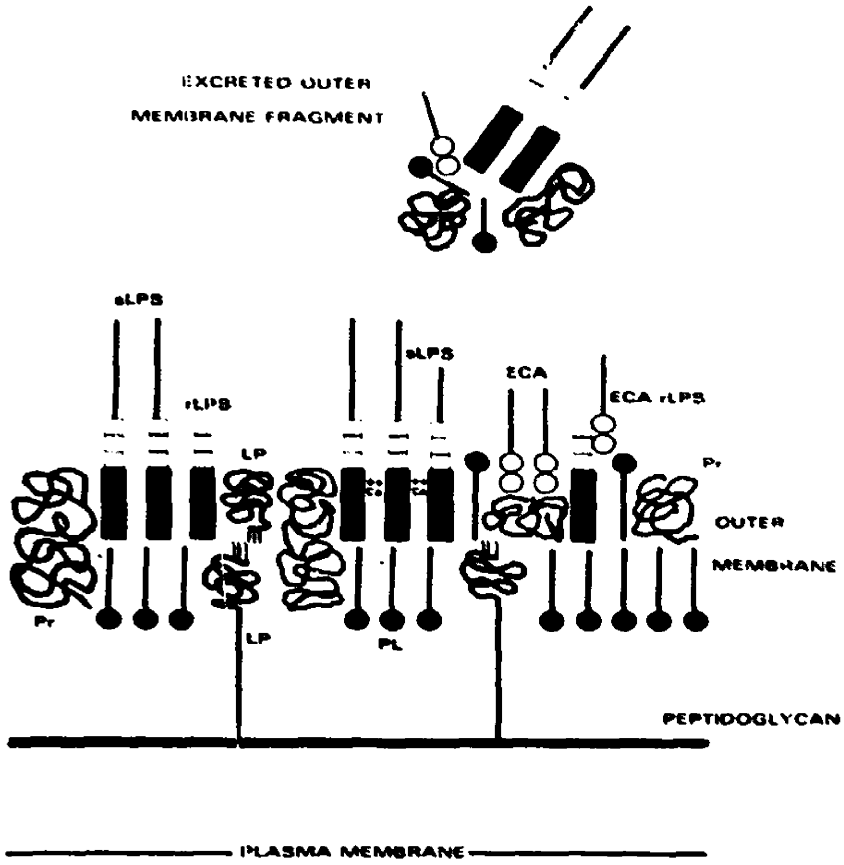

Fig. 2. Diagrammatic representation of a generalised Gram-negative bacterial cell envelope. The inner leaflet of the bilayer of the outer membrane is shown to be composed essentially of phospholipid (PL) and protein (Pr) with lipoprotein (LP) forming a covalent attachment to the peptidoglycan layer overlying the plasma membrane. In the outer leaflet of the outer membrane bilayer lipopolysaccharide (LPS) molecules are shown, the three regions of the lipopolysaccharide molecule are depicted as black rectangle (lipid A), open squares (core polysaccharide) and black bar (O-polysaccharide). Smooth and rough variants of lipopolysaccharide molecules are shown as sLPS and rLPS respectively. The enterobacterial common antigen is shown in its haptenic form (ECA) and also attached to a rough form lipopolysaccharide in its immunogenic form (ECA–rLPS). The possible presence of some of the lipoprotein (LP) complement in the outer leaflet (see text) is also allowed for. An excreted outer membrane fragment composed of lipopolysaccharide, enterobacterial common antigen, phospholipid and protein is depicted as a micellar aggregate in the external environment.

membrane movement can occur with lipopolysaccharide being found on both sides of the outer membrane [54]. It is therefore suggested that in the native state lipopolysaccharide is finally located randomly dispersed in the outer leaflet of bilayered outer membrane and covers approx. 30–40% of its area [55]. Phospholipids on the outer hand are believed to be concentrated in the inner leaflet of the bilayer [13,56]. In contrast to *S. typhimurium* it has been demonstrated in *E. coli* that the distribution of lipopolysaccharide is not random but rather in domains that do not intermix freely with each other and may be of different composition [57]. It was earlier shown that EDTA treatment of *E. coli* could release up to 50% of the lipopolysaccharide [58] indicating two forms of the polymer in

the outer membrane. Newly synthesized lipopolysaccharide was not EDTA-extractable but with time became distributed between EDTA-extractable and non-extractable forms. It has been suggested [59] that a fraction of the lipopolysaccharide molecules are bound to each other through divalent cations (EDTA-extractable) while the other fraction interacts hydrophobically or ionically with other molecules in the membrane.

Enterobacterial common antigen is presumed to occupy a similar location in the outer membrane to lipopolysaccharide particularly in those strains where it is linked to R-form lipopolysaccharide stubs [11].

Lipoprotein, on the other hand, or most of it, is inserted in the inner leaflet of the outer membrane [12,13]. A third of it is covalently linked to the underlying peptidoglycan and provides anchor points for the outer membrane to the more rigid peptidoglycan [12,13]. Some exposure to the outer surface of at least a portion of the lipoprotein is suggested by immunological studies [12,12a]. In smooth strains, with complete lipopolysaccharides, lipoprotein was non-immunogenic but in rough mutants, lacking the shielding O-specific side chains, lipoprotein was immunogenic and could be detected at the surface with specific antisera [12,12a].

There have been numerous reports of the release of outer membrane fragments in the form of lipopolysaccharide-phospholipid-protein complexes from growing and stationary phase cells [60–67]. In *E. coli* this appears to be, preferentially, newly synthesized material [67]. Thus complexes containing mixtures of the Gram-negative bacterial amphiphiles can be found in the bacterial external environment. This has important connotations in considering the biological activities of these amphiphiles in eucaryotic systems. The possibility that Gram-negative organisms may in some cases excrete only the carbohydrate moiety of lipopolysaccharide is illustrated by the finding that *Xanthomonas* sp. formed an extracellular polysaccharide that had a sugar composition including ketodeoxyoctonate, reminiscent of lipopolysaccharide, but lacking lipid [68].

B. Gram-positive bacteria

Gram-positive bacteria have a much simpler wall structure. Surrounding the cytoplasmic membrane is a cross-linked network of peptidoglycan to which polysaccharides and teichoic acids are covalently bonded as the major components. The known and proposed relationships of Gram-positive bacterial amphiphiles to the wall-membrane complex are shown in Fig. 3.

The discovery of a membrane lipid component in lipoteichoic acids readily suggested an attachment of these amphiphiles to the plasma membrane by simple intercalation of the fatty acid residues of the lipid-moiety with the upper half of the bilayer of the membrane. Serological detection of lipoteichoic acid at the surface of some bacteria led to the proposal of a model [69] in which the long polar glycerophosphate chain of the lipoteichoic acid could penetrate the peptidoglycan network of the cell wall and, in some cases, be detectable as a surface antigen. This is the case for instance in *L. fermentum* where lipoteichoic acid is the group antigen and major surface immunogen when whole organisms are injected into rabbits [70]. Support for this model was obtained by the use of ferritin conjugated to goat antirabbit γ-globulin to detect the sites of interaction between lipoteichoic acid and rabbit antibody specific to the polyglycerophosphate chain [69]. Electron micrographs showed heavy labelling of the surface of whole organisms, protoplasts and membrane fragments while extension of the technique to immunochemical labelling after fixation and thin-sectioning [71] showed ferritin label extending from

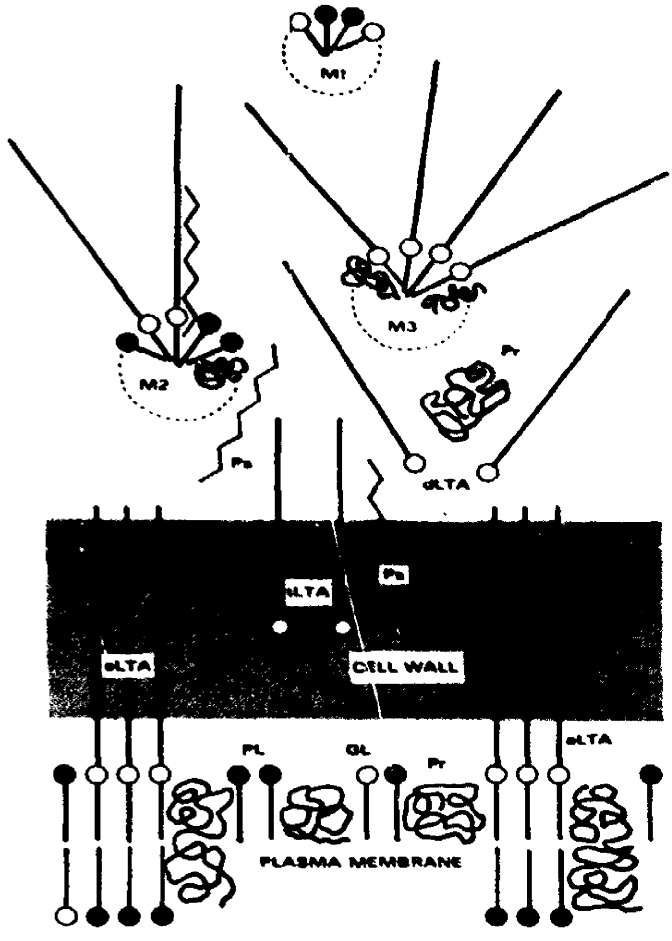

Fig. 3. Diagrammatic representation of a generalised Gram-positive cell wall-plasma membrane complex. The plasma membrane is shown as being composed of protein (Pr), phospholipid (PL), glycolipid (GL) and, in the upper leaflet of the bilayer, acylated lipoteichoic acids (aLTA), the hydrophilic polyglycerophosphate chains of which extend through the matrix of the cell wall to the cell surface. Lipoteichoic acid molecules in the process of excretion from the cell and having a transitory location in the cell wall (tLTA) are shown in both acylated and deacylated form. Wall polysaccharide (Ps) is also shown in the process of excretion. The external milieu is depicted as being composed of excreted polysaccharide, protein, deacylated lipoteichoic monomers (dLTA) and various micellar complexes of (a) excreted membrane lipids (M1), (b) acylated lipoteichoic acid, protein, phospholipid and polysaccharide (M2), and (c) acylated lipoteichoic acid and protein (M3). Other Gram-positive bacterial amphiphiles discussed in the text are not shown as their precise locations, while assumed to be similar to lipoteichoic acids, are not known.

the upper surface of the membrane, through the wall, to the surface of the cell as demanded by the model. Space-filling models of the *Str. faecalis* lipoteichoic acid [21] showed clearly that the spatial orientation of the four fatty acid hydrocarbon chains was such that the proposed intercalation into the upper half of the membrane bilayer was stereochemically possible and would act as a membrane 'anchor' for lipoteichoic acid.

These results suggest a generalized location of lipoteichoic acid over the surface of the plasma membrane. A more specific site of location entirely in mesosomes has been sug-

gested [72] from the analysis of mesosomal and plasma membrane fractions from *Staphylococcus aureus*. Similar studies with *Str. faecalis* [73] did not support this view. The dangers of assignment of specific locations for lipoteichoic acid or other amphiphiles on the basis of sub-cellular fractions alone is compounded by the ready loss and perhaps relocation of lipoteichoic acid from membranes under conditions of low Mg^{2+} concentrations [74]. It is of course possible that localised concentrations of lipoteichoic acid may occur in regions of the cell and/or at different times during the cell cycle.

More recently this somewhat static model of lipoteichoic acid location had to be modified with the finding that many organisms excreted lipoteichoic acid into the external environment [75,76] in significant quantities and under conditions where there was no wall turnover or cell lysis. A transient existence of lipoteichoic acid as a solely wall and surface component could also be envisaged [77] as a stage in the process of lipoteichoic acid excretion. As a further complication lipoteichoic acid can be recovered from both locations (extracellular and cellular) in two forms, a high molecular weight micellar aggregate or true amphiphile and a lower molecular weight deacylated monomer form which, lacking the fatty acid hydrocarbon chains, cannot undergo hydrophobic aggregation. The relative proportions of lipoteichoic acid in the two locations as well as the proportion of acylated micellar form to deacylated monomer varies widely with the species of organism and its phase of growth [75–77]. In *Str. faecalis* extracellular lipoteichoic acid is entirely in the deacylated monomer form during logarithmic growth and evidence has been presented that it is derived directly from the cellular lipoteichoic acid through the action of a membrane'deacylase' [78]. In many of the oral streptococci and lactobacilli, on the other hand, lipoteichoic acid is excreted in both molecular forms even under balanced growth conditions in the chemostat [77,79,80]. With *Str. mutans* BHT the amount of extracellular lipoteichoic acid is some 8–9-fold greater than the cellular lipoteichoic acid at pH 6.0 and generation times of 14 h [77,79,80]. *L. fermentum* shows a marked increase in excretion of lipoteichoic acid at generation times approaching that generally estimated to be likely growth rates of microorganisms in the nutritionally limited environment of the oral cavity [77,81]. Treatment of growing organisms with antibiotics such as penicillin or starvation for an essential amino acid also increases the excretion of lipoteichoic acid and lipids [82,83].

With regard to the other Gram-positive bacterial amphiphiles rather less is known of their precise location. The structural analogy of lipomannans to lipoteichoic acids suggests a similar location and lipomannan has been shown to be a major surface antigen of protoplasts of *M. luteus (lysodeikticus)* [25,84]. Some enrichment of the lipomannan has been reported in the mesosome fraction [25,84] and it appears to be more firmly bound to membrane than lipoteichoic acid, but this could be due to a lower hydrophilicity of the lipomannan compared to the highly charged lipoteichoic acid [84]. The *Actinomyces viscosus* amphiphile can similarly be detected at the cell surface and both amphiphiles have been found in culture fluids [27] although it is not known whether excretion involves deacylation in whole or in part.

VI. Biological properties

One of the most outstanding properties of amphiphiles is their ability to bind to erythrocyte membranes. All of the amphiphiles discussed above do this readily and generally without modification of the erythrocyte surface or amphiphile molecule [1,2,9,10,12,16, 27]. Agglutination of amphiphile-sensitized erythrocytes with antisera is used as a semi-

quantitative assay procedure [2,16,17,27]. It has also been used to detect and quantify lipoteichoic acids in culture fluids [75,80] and, since deacylated lipoteichoic acid monomers do not sensitize erythrocytes (vide infra), this method will distinguish between the acylated and deacylated forms of the amphiphile. Haemagglutination has also been exploited in the detection of new amphiphiles. Utilizing the principle that amphiphiles are generally surface located and immunogenic as well as present in culture fluids, *A. viscosus* was grown in the diffusible portion of dialysed medium, the organisms were used to immunize rabbits and the non-diffusible portion of spent media was used to sensitize erythrocytes. Subsequent haemagglutination to high titres indicated the presence of an erythrocyte-sensitizing species in the culture fluid which was then isolated and partially characterized as a new type of amphiphile [27].

Lipopolysaccharides, the best known amphiphiles, show a wide range of biological activities which have been studied for over a century (see for instance Refs. 1–6); the cellular location of this component and its toxic properties led to the alternate and older name of endotoxin. As shown in Table I, some of these properties are shared by the other classes of amphiphile. Broadly these properties can be grouped into: (a) those which involve binding of the effector amphiphile to a cell membrane, and (b) those involving intermolecular reactions in solution. The list is incomplete as many of the new amphiphiles still have to be tested for certain biological properties and the table is by no means exhaustive as far as the effects of lipopolysaccharide are concerned [5,6,50].

It should also be borne in mind that the conclusions regarding lipopolysaccharides are based on the studies with preparations from enterobacteria, particularly *Salmonella* sp. and *E. coli*, and do not necessarily apply to all lipopolysaccharides. Some examples of lipopolysaccharides that differ in their properties from enterobacterial lipopolysaccharides are:

(1) The preparation from *Chromobacterium violaceum*, which does not react with complement [85].

(2) The preparations from *Rhodospirillum tenue* and *Rhodopseudomonas viridis* which do not display lethal toxicity in adrenalectomized mice; *Rh. tenue* lipopolysaccharide also shows no reactivity with complement and is only weakly pyrogenic [85].

(3) The lipopolysaccharide of *Bacteroides asaccharolyticus* which does not give a dermal Shwartzman reaction and requires considerably higher doses than normal to give a positive reaction when tested for *Limulus* lysate gelation and chick embryo lethality [7].

Lüderitz and coworkers [85] have compared the structures of the lipid A component from *C. violaceum* and Rhodospirillaceae sp. with those from *Salmonella* sp. Differences in the lipid A backbones and in their substituents were evident, particularly in the case of *Rps. viridis* lipopolysaccharide, but all contained a 3-hydroxy alkanoic acid. The *Bacteroides* lipopolysaccharide has not been subjected to such a thorough study; it is atypical in that it lacks heptose, 2-keto-3-deoxy-octonate and D-3-hydroxymyristic acid [7] though whether another 3-hydroxy alkanoic acid is present is not known.

VIA. Binding of amphiphiles to eucaryotic cells

While all amphiphiles will bind to erythrocytes and other mammalian cells only two classes, namely lipopolysaccharides and lipoteichoic acids, have been examined in any detail.

VIA(1). Lipopolysaccharides

The lipid moiety (lipid A) of lipopolysaccharide is essential for membrane binding.

TABLE I

SOME BIOLOGICAL PROPERTIES OF AMPHIPHILES

Property	Lipopolysaccharide	Lipoteichoic acid	Enterobacterial common antigen	Lipoprotein	*Actinomyces* amphiphile	Lipomannan
Pyrogenicity	+	–	–	–		
Lethal toxicity	+	–	–	–		
Immunogenicity	+	+	+	+	+	+
Mitogencity	+	+		+	+	
Eucaryotic membrane binding	+	+	+	+	+	+
Stimulation of bone resorption	+	+			+	
Anticomplementary activity	+	+				
Stimulation alternate complement pathway	+	+				
Shwartzman reaction	+	+				
Hypersensitivity reactions	+	+				
Stimulation of the reticuloendothelial system	+	+				
Stimulation of non-specific immunity	+	+				
Macrophage lysosomal enzyme releas	+	+				
Limulus lysate assay	+	+			+	

Lipid-free polysaccharides do not bind to erythrocytes but esterification with as little as 5% *O*-stearoyl groups gives ready and optimal sensitization [86].

Identification of the eucaryotic membrane receptors has proved more difficult. Studies on the receptors of the erythrocyte membrane have employed both indirect and direct procedures. The indirect procedure, where compounds were examined for their ability to inhibit the binding of lipopolysaccharide, showed that phospholipids and cholesterol were active. An interaction between phospholipid and lipopolysaccharide has also been indicated by the specific requirement for certain types of phospholipid in in vitro biosynthetic systems [87]. Direct studies on the specific receptor led to the isolation from human erythrocyte membranes of a lipoglycoprotein, where the evidence suggested lipid A was binding to hydrophobic residues of the protein portion [88]. The conclusion that the isolated complex was the specific receptor was supported by the observation that different lipopolysaccharides competed for the same receptor, the receptor did not bind other antigens that were tested, and the receptor would remove lipopolysaccharide already bound to erythrocytes [88]. Studies on leucocyte-lipopolysaccharide interaction by the same laboratory [89] have given the suggestion that the receptor in this case is in a membrane phospholipid fraction. In both cases the receptors need to be more fully defined chemically to substantiate the author's suggestions of different receptors in the two cell types.

A recent study [90] on the binding of radiolabelled *E. coli* lipopolysaccharide to a variety of eucaryotic cell types showed a consistent pattern of adsorption and desorption which was interpreted as indicating membrane reorganization and heterogeneity in the lipopolysaccharide population. In the latter case the more hydrophobic molecules were suggested to penetrate the bilayer causing membrane reorganization and desorption of ionically bound less hydrophobic molecules at the surface.

As a means of studying the interaction of lipopolysaccharide with membrane, several investigators have employed model systems. Studies on the penetration of phospholipid monolayers by lipopolysaccharides [91–94] have also indicated a structural specificity, phosphatidylethanolamine layers being penetrated readily while phosphatidylcholine layers were not. Alkali treatment of the lipopolysaccharide, presumably resulting in some simplification of the lipid A region through removal of ester-linked but not amide-linked fatty acids, gave greater penetration of phospholipid monolayers and, unlike the native lipopolysaccharide, monolayers of cholesterol. Treatment of artificial bilayers of phosphatidylcholine and cholesterol with lipopolysaccharides gave a decreased stability of the bilayer but not if the amphiphiles were first deacylated with hydroxylamine [92]. Artificial liposomes will also incorporate lipopolysaccharides into their structure, and in the presence of anti-lipopolysaccharide antisera and complement will undergo immune lysis [95]. These results reinforce the idea of essentially lipid-lipid attraction in lipopolysaccharide-cell membrane interactions and the possibility of unstabilizing effects on cell membranes could be the trigger for various biological effects of lipopolysaccharides on cells. If lipopolysaccharides will only interact with certain phospholipids, then variation in phospholipid composition from one type of eucaryotic cell to another could explain some of the specificity of the lipopolysaccharide-triggered reactions [6].

VIA(2). Lipoteichoic acids

As with lipopolysaccharides, most detailed studies of lipoteichoic acid binding to eucaryotic cells have been carried out with erythrocytes. Spontaneous binding of lipoteichoic acid to oral mucosal cells, human platelets and a variety of other mammalian

cells [96–99] have also been reported. In all cases the lipid moiety appears essential for binding in that the deacylated molecule is no longer active whether this be prepared by mild alkali treatment [16,97] or digestion with *Candida cylindracea* lipase (Wicken, A.J. and Knox, K.W., unpublished observations) or isolated as naturally occurring monomer. To demonstrate further the role of the fatty acid substituents in this process Beachey et al. [100] used [^{3}H]lipoteichoic acid from *Str. pyogenes* as a more sensitive test of erythrocyte binding than haemagglutination with anti-lipoteichoic acid antisera. Mild alkaline deacylation removed, as expected, the binding capacity but this could be restored to nearly 50% of the original by re-esterifying with stearoyl chloride in *N,N*-dimethylformamide and pyridine. Treatment of the deacylated lipoteichoic acid with the various reagents in the absence of stearoyl chloride did not restore the binding capacity. Thus the main body of evidence would support strongly the contention that the fatty acid substituents of lipoteichoic acids are essential for their ability to adsorb to the erythrocyte surface and is completely contrary to the idea [101] that lipid is non-essential to the process.

Binding of [^{3}H]lipoteichoic acids from *Str. pyogenes* or *Str. faecalis* to human and sheep erythrocytes has been found to be cell concentration, time and temperature dependent [100]. At 37°C binding approached a maximum only after 2 h of incubation. Estimation of the average number of lipoteichoic acid receptor sites per adult human erythrocyte as well as their binding affinity gave values of approx. $29 \cdot 10^6$ sites with a dissociation constant of 4.5 μM; human cord blood erythrocytes gave figures of $30 \cdot 10^6$ sites but a higher dissociation constant of 31 μM, while sheep erythrocytes showed $7.2 \cdot 10^6$ sites and a dissociation constant of 1.6 μM.

Evidence that the binding is reversible, as with lipopolysaccharide binding, was provided [100] by 'cold-chase' experiments using excesses of homologous and heterologous lipoteichoic acids. The binding of radiolabelled lipoteichoic acid could also be inhibited by an excess of unlabelled heterologous as well as homologous lipoteichoic acid [100]. These results indicate that similar receptor sites on the erythrocyte surface are involved in the binding of lipoteichoic acids irrespective of their structure. This is not unexpected, given a hydrophobic interaction, when the overall heterogeneity of the fatty acid substituents of lipoteichoic acids are considered, i.e. probably lipoteichoic acids from different species are very similar as far as their hydrophobic 'ends' are concerned.

In contrast, lipopolysaccharides from *Salmonella enteritidis* or *Serratia marcescens* failed to inhibit the binding of radiolabelled lipoteichoic acid or affect the kinetics of binding, indicating that these two amphiphiles have different binding sites [100]. Evidence that both amphipathic species were bound to the same erythrocyte was provided by haemagglutination with antisera specific for their amphiphile when the titre was the same as with erythrocytes sensitized with either amphiphile alone. Binding of lipoteichoic acid to human erythrocyte membranes only affected accessibility of A and B bloodgroup antigens to their respective antibodies as occupation of lipoteichoic acid binding sites approached saturation [100].

Studies on lipoteichoic acid binding sites on the erythrocyte point to importance of protein components rather than carbohydrates [100,102]. Binding of lipoteichoic acid to erythrocyte ghosts showed similar characteristics to whole cell binding and SDS gel electrophoresis indicated lipoteichoic acid association with a single protein band, distinct from that associated with lipopolysaccharide receptors [102]. Further work is required to determine whether this protein association is real, and represents a true receptor, or fortuitous through the extraction procedure.

The hydrophobic component of lipoteichoic acid is of course simpler in structure and closer to eucaryotic membrane lipids than that of lipopolysaccharides and binding through intercalation into the membrane bilayer is probably easier in the former than in the latter case. It was early observed that alkali-treated, with subsequent loss of some fatty acids and bilayer structure, lipopolysaccharide binds more readily to membranes than the native form [103]. The demonstrated associations of lipopolysaccharide and lipoteichoic acids with distinct erythrocyte membrane proteins may well be a secondary event following a primary lipid-lipid hydrophobic interaction. It has been noted [104] that alkali-treated lipopolysaccharide not only has a greater affinity for erythrocytes but will also cause haemolysis under certain conditions in the absence of antibody or complement. Lipoteichoic acid like native lipopolysaccharide on the other hand does not cause haemolysis and this may reflect a greater penetration and perturbation of the lipid bilayer of the membrane in the case of alkali-treated lipopolysaccharide. It is perhaps a pity that lack of toxicity of lipoteichoic acid could not be explained by a lack of membrane perturbation since haemolytic alkali-treated lipopolysaccharides are generally less toxic than the native form [92,105].

The binding of lipoteichoic acid to eucaryotic cell membranes has also been proposed as a factor contributing to the binding of group A streptococci to several types of mammalian cells [106]. Plasma membrane glycolipids can mediate eucaryotic cell-cell adhesion [107]. However, the evidence presented thus far [106] suggests that the reaction between the streptococcal and eucaryotic cells is more complex than a simple binding of lipoteichoic acid, and probably involves protein components and both ionic and hydrophobic forces [18].

The ionic properties of lipoteichoic acid have been suggested as playing an important role in another important adherence reaction, namely the adherence of bacteria to the tooth surface to form dental plaque [75,108]. This conclusion is based on the observation that lipoteichoic acid will bind readily to hydroxyapatite, presumably through ionised phosphate groups and divalent cation bridging [75].

VIB. Consequences of amphiphile-eucaryotic cell interaction

The plethora of biological effects of amphiphiles in eucaryotic systems has already been referred to and a summary of some of these properties is shown in Table I. Some of these effects are toxic, leading to the death of the organism whereas some may be considered beneficial.

VIB(1). Pyrogenicity and lethal toxicity

Thus far the classical endotoxic properties of pyrogenicity and lethal toxicity evinced by lipopolysaccharides appear to be restricted to members of this class of amphiphile. The lipid A moiety of lipopolysaccharides has been shown [50,85] to be responsible for these toxic effects and isolated lipid A will produce most of the biological effects of native lipopolysaccharide. The lack of endotoxic-like effects of lipoteichoic acids (non-lethal in mice in doses up to 100 mg/kg body weight and non-pyrogenic in rabbits [109]) probably relates to the much simpler lipid substituents of lipoteichoic acids and lack of hydroxyacyl esters characteristic of lipid A. Lipoprotein [12] and the enterobacterial common antigen [136] are similarly non-toxic and non-pyrogenic. The other classes of amphiphile have not been examined in this connection but it would seem likely that these too, with their simpler lipid components, will prove also to be non-pyrogenic and non-

toxic. The mechanism of endotoxic effects of lipopolysaccharides is still poorly understood. There is some evidence that activation of membrane-bound adenyl cyclase systems is involved with alterations in cellular levels of cyclic AMP and cyclic GMP [6]. Apart from pyrogenicity and lethality, the Shwartzman phenomenon, bone-marrow necrosis, leucopenia, leucocytosis, hypotension, abortion and tumour necrosis can be considered to be toxic properties of lipopolysaccharides [6]. It is now becoming apparent that with the general activation of so many eucaryotic systems the observed physiological effects are likely to be due to combinations of stimulation of different systems and that it is the concurrent overstimulation of a range of processes in the host that results in the toxic events observed [6]. In other words toxicity results from activation of various cell types rather than from a direct toxic effect of the lipopolysaccharide molecule [6].

VIB(2). Immunogenicity

The detection of all known amphiphiles as surface antigens has already been referred to. Their significance as major or minor surface antigens varies with the species of organism and class of amphiphile as also does their immunogenicity. Immunogenicity of isolated amphiphiles is very dependent on mode of presentation and physical state of the amphiphile. As with other biological properties the most studied immunogenic effects of amphiphiles are restricted to lipopolysaccharides [1–4,50,85] and lipoteichoic acids [16, 17,77,79,109]. Space does not permit a detailed discussion of a wealth of literature on this topic which has been well reviewed in the references cited above. However, some of the salient features and differences between the immunogenic and antigenic properties of lipopolysaccharides and lipoteichoic acids are worth summarizing.

Lipopolysaccharides are well known as thymic independent immunogens that induce polyclonal B cell activation with a predominantly IgM humoral response. Either on the bacterial cell surface or following isolation (especially if the preparations contain bacterial protein) they are generally potent immunogens. Antibody specificity is generally directed at determinants on the polysaccharide portion of the molecule (immunodominant sugars are the basis for the serological classification of many Gram-negative bacteria). Potent anti-lipid A antibodies can be obtained by injection into animals of deep-rough mutants from which ketodeoxyoctonate has been removed and whose surface has been coated with excess lipid A, and such antisera are widely cross-reactive [50].

Lipoteichoic acids, on the other hand, appear to be T-cell dependent immunogens and their humoral response gives rise to both IgM and IgG antibodies [16,17]. Specificity is variously directed against the glycosidic substituents, or the polyglycerophosphate backbone of the molecule, which is common to all lipoteichoic acids, with the result that the antibodies are cross-reactive. The immunogenicity of lipoteichoic acids depends on the method of presentation. In some organisms there is sufficient exposure of lipoteichoic acid at the cell surface for it to be expressed as a major immunogen when whole organisms are injected; in other organisms immunogenicity is increased or may only be detectable if disrupted organisms are injected [16,17]. The immunogenicity of isolated lipoteichoic acids is variable and dependent at least to some extent on the amount of complexed protein present in the preparation [40] and the use of adjuvants, such as Freund's complete adjuvant, is generally required to give antisera of high titre [16,17,109]. Deacylated monomer lipoteichoic acids are non-immunogenic unless complexed with other substances with a resultant higher molecular size or particulate form.

Lipoprotein has been reported to give a predominantly IgM response following injection of rough mutants, where lack of *O*-specific polysaccharide allows its exposure at the

cell surface, or of lipoprotein-coated erythrocytes [12]. Antigenic specificity appears to be mainly in the C-terminal or non-lipid end of the molecule [12,137]. Data concerning the immunogenicity of the other amphiphile classes are restricted mainly to humoral responses following injection of whole organisms [10,11,25,27,84] and specificity generally relates to the carbohydrate portions of their molecular structure.

Hypersensitivity reactions to lipoteichoic acids have been observed in both our laboratories (Wicken, A.J. and Knox, K.W., unpublished observations) and that of Fiedel and Jackson [110]. In both cases the reactions observed are complex and clear-cut definitions of the types of hypersensitivity involved are not possible at this stage except that it seems unlikely that cell-mediated type IV hypersensitivity is involved. In rabbits immunized with complexes of streptococcal group A lipoteichoic acid with bovine serum albumin, hypersensitivity reactions with the properties of both type I and type III hypersensitivity were observed [110]. In our laboratories (Wicken, A.J., Knox, K.W., Jackson, D. and Jackson, G.D.F., unpublished observations) anaphylactoid symptoms and Arthus-type reactions have been observed in rabbits immunized and challenged with different doses of lipoteichoic acid and bacterial cells with surface-associated lipoteichoic acid.

VIB(3). Mitogenicity

Mitogenic stimulation of lymphocytes by immunogens either directly or in admixture with adjuvants appears to be a major factor in eliciting the immune response. Lipopolysaccharides are well-established as potent B- but not T-lymphocyte mitogens and will act as adjuvants for other immunogens, presumably through a similar B-cell activation. That lipid A but not deacylated native lipopolysaccharides will act similarly is indicative of the importance of the hydrophobic portion of the molecule in this activation. Binding of lipopolysaccharide to lymphocytes while presumably a prerequisite for activation is not sufficient in itself for activation since binding is the same in lymphocytes from spleens of mice strains that are (a) responsive, and (b) unresponsive to lipopolysaccharide-induced mitogenesis [6,111]. Lipoprotein has similarly been reported to be a B-cell mitogen and T-independent with release of ester-linked fatty acids resulting in loss of mitogenicity [12]. The mitogenic status of the other amphiphile classes, particularly lipoteichoic acids, is still far from clear. Evidence that lipoteichoic acids can suppress the immune response to sheep erythrocytes in mice but stimulate the response to lipopolysaccharide is suggestive of some effect on the antigen processing section of the immune system [112]. Initial studies on isolated lymphocytes suggested no mitogenic activity for lipoteichoic acids [17,112]. More recently conflicting claims that lipoteichoic acids are exclusively T-cell [113] or B-cell mitogens (Chen, U. and Mishell, R.I., personal communication) have created some confusion. In both cases the degree of stimulation, while dose-dependent, is not as marked as with lipopolysaccharides. The conflict as to which lymphocyte type is stimulated, since both T- and B-lymphocytes appear to bind lipoteichoic acids [113], probably relates to differences in lymphocyte preparation and homogeneity while the significance of the stimulation may be questioned on the grounds of purity of the lipoteichoic acid preparations used, i.e., are the relatively low levels of stimulation observed due to lipoteichoic acid or perhaps complexed protein? Whatever the answer the present situation is a clear indication of the caution required in interpreting results in this area and the need for fully standardised test systems as well as clear criteria of purity of the preparations under test. In this connection it is worth noting that phenol/water preparations (containing approx. 1% protein) of *Salmonella abortus equi* lipopolysaccharide showed a low mitogenic activity in C3H/He J mice (lipopolysaccharide-resistant) while

the protein-free Novo-Pyrexal preparation (see Section IIIC) was completely free of mitogenic activity. Both preparations were highly mitogenic in normal mice [42].

VIB(4). Effects on other eucaryotic cellular systems

(a) Bone resorption. The phenomenon of bone resorption and consequent loss of alveolar bone is of considerable significance in advanced periodontal disease. Test systems involving the measurement of ^{45}Ca release from rat fetal bone in organ culture show stimulation of the process by lipopolysaccharides, lipoteichoic acids [114] and the preparation of *Actinomyces* amphiphile (Hausmann, E. and Wicken, A.J., unpublished observations); in all cases an intact lipid moiety in the amphiphile is essential for activity. The importance of fatty acid esters is supported by observations that bone resorption is also stimulated by free fatty acids [115] and prostaglandins which can be considered as fatty acid derivatives [116]. The mechanism of the stimulation may not be the same in each of the amphiphile classes studied (Hausmann, E., personal communication).

(b) Macrophage stimulation. Both lipopolysaccharides and lipoteichoic acids will stimulate macrophages as measured by increases in the phagocytic index in carbon clearance by the reticulo-endothelial system [6,17,50,109,110] or more specifically by lysosomal enzyme release [117] (O'Grady, R.L., Harrop, P.J., Wicken, A.J. and Knox, K.W., unpublished observation). However, lipoteichoic acid will only stimulate the release of preformed lysosomal enzymes (O'Grady, R.L., personal communication) whereas lipopolysaccharide also stimulates the synthesis of lysosomal enzymes and of collagenase [117]. Both classes of amphiphile stimulate non-specific immunity [4–6,16,17,109] which may be related, at least in part, to macrophage activation.

(c) Shwartzman reaction. The well-known Shwartzman reaction, in both its localized and generalized form, is produced in rabbits by lipopolysaccharides and lipoteichoic acids though somewhat higher doses of lipoteichoic acid are required [4,5,109,118]; in both instances the generalized reaction is accompanied by bilateral necrosis of the kidneys. The mechanism of the reaction is still obscure but it is generally accepted to involve neutrophil activation with release of lysosomal enzymes [118].

(d) Tumour necrosis. There have been numerous reports of tumour necrotizing properties of lipopolysaccharides [119–121] and the reaction once again appears to be associated with the hydrophobic portion of the molecule, lipid-rich endotoxins, viz. those from rough strains, possessing greater tumour necrotizing potential [122]. Neoplastic transformation has been associated with changes in cell membrane properties [123] and in this study the binding of a rough form lipopolysaccharide to rat fibroblasts was shown to be greater in transformed cells than in the normal cell line. In a similar context lipoteichoic acid from group A streptococci has been shown to have marked cell destructive capability for human kidney and liver cells in tissue culture; in this case, also, activity depended on hydrophobic interaction as deacylated material was inactive [124].

VIC. Molecular interactions of amphiphiles

VIC(1). Complement

While probably all amphiphiles are capable of activating complement by the classical pathway following antigen-antibody interaction, the direct reaction of amphiphiles with components of the complement system is a more specialized property, which has been studied in detail with lipopolysaccharide [4–6,50,125] and which has recently been shown for group A streptococcal lipoteichoic acid [126]. Inhibition of complement-

mediated lysis of erythrocytes by lipoteichoic acid [127] could be dependent on the consumption of complement by the alternate pathway or chelation of divalent cations necessary in the early stages of the classical pathway.

The generalization that lipopolysaccharides activate complement by the alternate pathway requires qualification. As indicated by the studies of Galanos and coworkers [85, 125], most lipopolysaccharides, whether from smooth or rough strains, are very active provided they are highly aggregated, as originally isolated, or after their conversion to a uniform salt (sodium or calcium salt). However, lipopolysaccharides present in a disaggregated form (e.g. triethylamine salt) do not react with complement and a number of lipopolysaccharides including ones from certain rough *Salmonella minnesota* strains and other genera mentioned previously [85] do not show any reactivity. It is also noteworthy that the selective abolition of anti-complementary activity by conversion of lipopolysaccharide to the triethylamine form, did not affect other endotoxic properties, and thus provided the first example of the selective dissociation of one biological activity from others exhibited by lipopolysaccharides [85,125].

VIC(2). Limulus lysate interaction

The gelation of lysates of amoebocytes from the crustacean *Limulus* by low concentrations (ng/ml) of lipopolysaccharides has been proposed as a simple and cheap method of assaying both pharmaceutical products and human body fluids for the presence of lipopolysaccharide [128,129]. However, the test is not as specific as has been suggested. Positive reactions are given by lipoteichoic acid [130] and the *Actinomyces* amphiphile (Wicken, A.J., Shockman, G.D. and Rudegeair, D.A., unpublished observations) though considerably higher concentrations are required. These results, together with the observation that deacylated lipoteichoic acid is inactive [130] could be interpreted as indicating that the reaction is another example of the unique reactivity of amphipathic molecules. However, peptidoglycan is active [103] and so also is a synthetic preparation of dextran phosphate [131]. The examination of the latter compound was part of a study on dextran derivatives in which it was shown that palmitoyl dextran, palmitoyl dextran phosphate and alkali-treated (deacylated) palmitoyl dextran phosphate were all active [131]. Thus while the *Limulus* test may be more sensitive to lipopolysaccharide it is not an exclusive property of any particular class of compounds.

VIC(3). Interactions with proteins and polysaccharides

Evidence that amphiphiles readily form complexes with other polymers such as proteins and polysaccharides has already been referred to and indeed presents the greatest problem in purification of amphiphiles. In vivo, however, such complexes are more likely to be the norm and the question must be asked as to what modulating effect the formation of such complexes may have on the biological effects of the individual components in vivo. Information in this area is at present sparse and confined largely to in vitro observations. Interaction of lipopolysaccharide and lipoteichoic acids with the protein components of complement would be one example of modulation of biological activity by amphiphiles. It is known that lipoteichoic acids and the pneumococcal F antigen can act as powerful inhibitors of bacterial cell wall autolytic enzymes [28,132–134]. Mammalian lysozyme, an enzyme common to many body fluids and reputedly having a beneficial role in controlling bacterial multiplication, is similarly inhibited by lipoteichoic acid (Cleveland, R.F., Wicken, A.J., Daneo-Moore, L. and Shockman, G.D., unpublished observa-

tions). These effects are lost upon deacylation of the amphiphile and are thus presumably hydrophobic in origin.

Dextransucrases from *Str. mutans* have been reported to be stimulated by the presence of phospholipids from human sera and oral fluids [135]. The level of activity of this enzyme in vivo has been related directly to the cariogenicity of *Str. mutans* and it is possible that lipid stimulation of activity may be of bacterial origin since many oral streptococci excrete lipids as well as lipoteichoic acid in significant quantities (Pieringer, R.A. and Shockman, G.D., personal communication). In *Salmonella* a clear requirement for and stimulation by phosphatidylethanolamine with respect to at least two of the glycosyltransferases involved in lipopolysaccharide synthesis has been elegantly demonstrated [91].

Interaction of amphiphiles with polysaccharides is evidenced by the difficulty in purifying certain lipoteichoic acids, for instance those from *Str. mutans* strains BHT [80] and AHT. In the latter case the native extracellular polysaccharide-lipoteichoic acid complex was capable of sensitizing erythrocytes, which were then agglutinated by polysaccharide-specific antiserum [44].

Such interaction of amphiphiles with other molecules can be envisaged as increasing the biological activity of the complex beyond that of the individual components. The proposed role of lipoteichoic acid in the binding of group A streptococci to mammalian cells provides one such example [18,106]. This phenomenon points to the need for more detailed investigations of the molecular interactions of amphiphiles with each other and with components present in their natural environment.

VII. Pathogenic potential of amphiphiles

The role of lipopolysaccharides in mammalian and human disease through direct or indirect cytotoxic effects, while still not clearly understood, has been clearly established. The purpose of this review article has been to underline the existence of other classes of amphiphile, particularly as excreted bacterial products, with many properties in common with lipopolysaccharides and which may also mediate in disease processes. Such mediation could be through:

(1) Immunogenicity, cross-reactive antibodies and hypersensitivity. (2) Mitogenic lymphocyte stimulation. (3) Complement activation by the classical and/or alternate pathways with the release of inflammatory factors. (4) Monocyte and macrophage activation with the release of lysosomal enzymes. (5) Stimulation of bone resorption as occurs in periodontal disease. (6) Involvement in the adherence of pathogens to specific cellular sites. (7) Stimulation or inhibition through direct molecular interactions of soluble factors such as enzymes involved in defense mechanisms of the host.

While the evaluation of real as opposed to potential roles of amphiphiles in disease is still largely in the future and will undoubtedly stimulate much research interest in this group of bacterial products, one common and important property of all amphiphiles has already emerged. That is the essential requirement for a hydrophobic lipid moiety in the molecule for the expression of practically all of the biological effects that have thus far been observed.

Acknowledgements

The authors' work described in this article has been supported by the Australian National Health and Medical Research Council and by U.S. Public Health Service grants

DE 04174 and DE 04175. We also acknowledge gratefully the collaboration of and the stimulation of discussion with many of our friends and colleagues in several parts of the worlds.

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