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The bacterial outer membrane as a drug barrier

Robert E.W. Hancock

The outer membranes of Gram-negative bacteria constitute a semi-permeable barrier, as indicated by the corresponding alterations in outer membrane permeability and in antibiotic susceptibility resulting from mutation or polycation action. Restricted outer membrane permeability works in synergy with co-determinant resistance mechanisms, such as the periplasmic enzyme β-lactamase or active efflux mechanisms, bringing about antibiotic resistance.

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Gram-negative bacteria have two cell-envelope membranes: an outer and a cytoplasmic membrane. Earlier work suggested that outer membranes are involved in the high intrinsic resistance of Gram-negative bacteria to antimicrobial drugs[1,2]. However, many recent reviews and papers[3,4] have emphasized efflux pathways, which are associated with the cytoplasmic or both membranes, as being critical for both intrinsic and multiple antibiotic resistance. These two apparently conflicting perspectives are in fact quite consistent because low outer membrane permeability and efflux are co-determinants of intrinsic resistance.

Structure of the outer membrane

Bacterial outer membranes have quite different compositions compared with most biological membranes, including bacterial cytoplasmic membranes. Normally, membranes comprise lipid bilayers with a restricted asymmetric distribution of lipids and a large variety of protein species 'floating' within this membranous matrix. In contrast, outer membranes generally consist of an almost completely asymmetric, conpositionally distinct bilayer, studded with a restricted number of protein species that are present in high copy number. (For a detailed discussion of outer membrane composition, structure and natural variants, see Ref. 5.)

The enterobacterial outer membrane bilayer consists of an inner monolayer containing phospholipids (primarily phosphatidyl ethanolamine) and an outer (surface) monolayer that largely consists of a single glycolipid species: lipopolysaccharide (LPS; Fig. 1). LPS is a high-molecular-weight, strongly negatively

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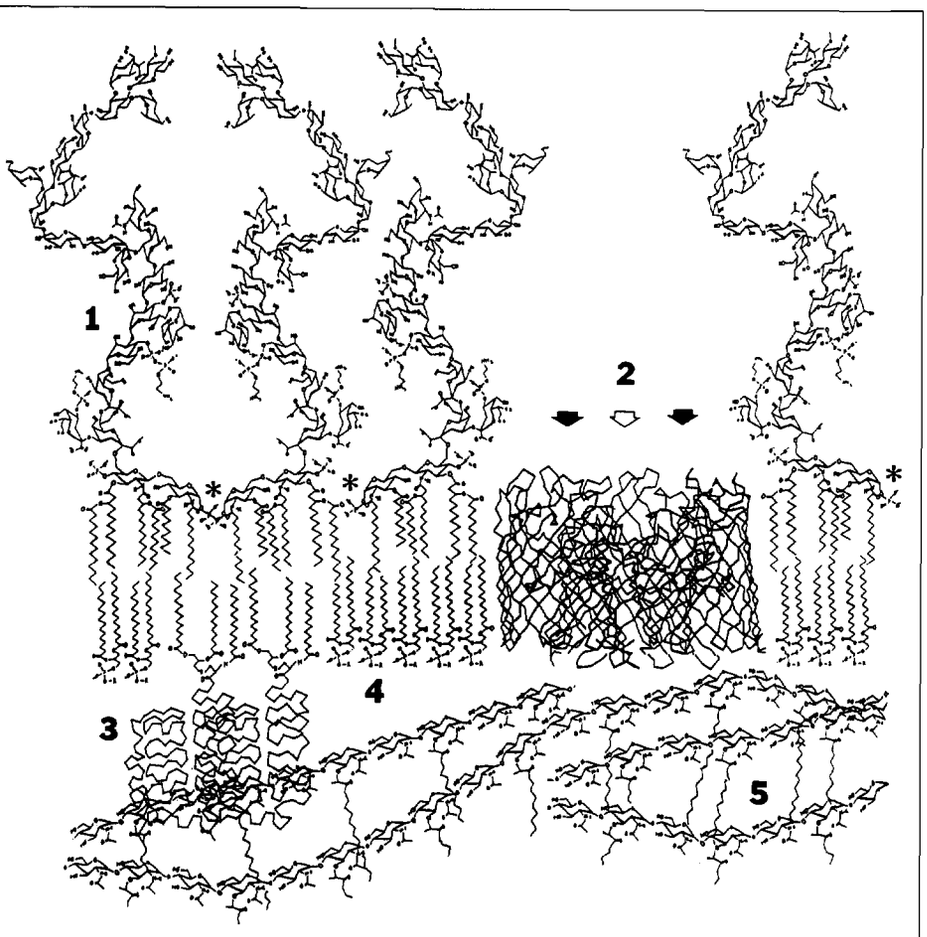

Fig. 1. A to-scale chemical model of a cross section of part of the *Escherichia coli* outer membrane. (1) Lipopolysaccharide (LPS), (2) matrix porin OmpF, (3) Braun lipoprotein, (4) phospholipids, (5) peptidoglycan, and the outer membrane stabilizing binding sites for divalent cations (asterisks) are shown. The structure of LPS shows two O-polysaccharide units; however, LPS can contain up to 40 of these pentasaccharides. The structure of OmpF shows a section of the trimeric porin, with two channels in the front (solid arrows) and one in the back (open arrow). The lipid component of the lipoproteins (that may be part of a trimeric arrangement) is inserted into the inner leaflet of the outer membrane, and the carboxy-terminus is linked (covalently or noncovalently) to the peptidoglycan layer, which consists of crossbridged *N*-acetylmuramic acid–*N*-acetyl glucosamine-tetrapeptide units. For clarity, only the amino acid backbones of the crossbridging peptide chains of peptidoglycan, of OmpF and of lipoprotein are shown. Phosphatidylethanolamine is the major lipid component of *E. coli* outer membrane, but other phospholipids, such as phosphatidylglycerol and cardiolipin, are also found. Reproduced, with permission, from Ref. 5.

charged molecule, which for smooth LPS can be divided into three regions: the lipid A portion of LPS inserts into the membrane, and in many Gram-negatives is diglucosamine diphosphate with 5–7 fatty acids attached (including unique hydroxy fatty acids). The lipid A is appended to a region (the rough core) of 8–12 variable sugars (including the unique negatively charged octasaccharide 2-keto-3-deoxyoctanate) and 3–8 phosphate residues, and this is covalently associated with the O antigen, which consists of 3–5 sugar units that are repeated a variable number of times. Mutants lacking the O antigen are called rough mutants because of their characteristic colony morphology, whereas in bacteria that naturally lack the O antigen, LPS is called lipo-oligosaccharide.

There are two dominant features of outer membrane structure that influence its functioning as a selective permeability barrier. The first is provided by proteins termed 'porins', which form water-filled channels that function as general or substrate-selective conduits for diffusion of certain hydrophilic molecules. The second is the high net negative charge on LPS molecules, which provides a polyanionic external surface that is partly neutralized by divalent cations (primarily Mg^{2+} and Ca^{2+}) and, for the purposes of discussion, can be considered to bridge adjacent LPS molecules. This feature, in combination with efflux, is probably responsible for the high resistance of the outer membrane to externally added detergents and dyes.

Antibiotic-uptake pathways

When considering the uptake of small hydrophilic molecules, the outer membrane is often described as a molecular sieve, in which the fabric of the outer membrane is considered impermeable and the holes of the 'sieve' are provided by the porins. Although this analogy is rather crude, and in some senses quite inaccurate (see below), it remains a reasonable analogy for small hydrophilic molecules. Crystal structures are now available for five of these porins[6–8] and show trimers of β-barrels consisting of antiparallel β-strands that pass through the outer membrane at a small incline to the perpendicular. In each of the five porins, the actual number of β-strands is 16 or 18; however, based on molecular genetic analyses[9], between 8 and 32 strands have been suggested for other outer membrane proteins. The β-strands are connected at the periplasmic side by short stretches of amino acids forming a β-turn, and at the external side by longer 'loops'. The longer-loop residues either fold back into the channel to form the most constricted portion of the channel (loop 3), reach over to contact an adjacent monomer (loop 2) in the porin trimer, or are found at the surface (mouth) of the channel. Porins have been suggested to fit into three classes[2,9,10]: general porins that have minimal substrate selectivity, specific porins that are substrate selective by having a specific binding site within the channel, and gated specific porins that have channels that are normally inaccessible but which are presumed to open upon binding of a specific substrate. Gating of porins[9] has not been proven; however, the demonstrated binding of the substrate to the porin, the

proven involvement of the porin in substrate uptake, based on mutational studies, and the functioning of the porin in salt uptake after deletion of the presumed 'gating' loop is very strong suggestive evidence. Although it is unusual, the substrate-specific and gated porins are known, in specific instances, to be involved in antibiotic uptake; for example, the basic amino-acid-specific porin OprD of *Pseudomonas aeruginosa* is also a specific channel for the β-lactam imipenem, and the iron-scavenger (gated?) channels Cir and Fiu of *Escherichia coli* are known to serve as channels for catechol β-lactams. However, the predominant channels involved in antibiotic uptake are the general porins.

The details of the uptake of hydrophobic molecules across outer membranes are poorly understood[11,12]. It was previously assumed that the high resistance of most Gram-negative bacteria to hydrophobic antibiotics indicated that the outer membrane constituted a powerful exclusion barrier to such substances. However, it is now clear that mutants defective for specific efflux pathways, without any known outer membrane barrier alterations, are considerably more susceptible to hydrophobic antibiotics in general. Studies of steroids have suggested that the outer membrane bilayer shows at least 10–100-fold slower rates of permeation compared with phospholipid bilayers[13]. It appears that outer membranes slow the passage of hydrophobic antibiotics, although not as much as previously thought, presumably because the highly charged surface and the stabilization of this surface by divalent cations inhibit partitioning of these antibiotics into the hydrophobic interior of the bilayer.

A third uptake system is termed self-promoted uptake and involves uptake of polycationic antimicrobials, such as aminoglycosides, polymyxins and natural polycationic peptides[5,12]. Such molecules interact with divalent cation binding sites on surface LPS molecules and, because they have an affinity for these sites that is 2–4 orders of magnitude higher than these divalent cations, they competitively displace them. The bulkiness of the displacing polycations leads to a distortion of outer membrane structure (electron microscopy reveals this as blebbing) and consequent permeabilization of the outer membrane to a variety of compounds, including hydrophobic molecules, various antibiotics and even the protein lysozyme. At the same time, uptake of the polycations themselves is enhanced, leading to the name 'self-promoted' uptake.

Importance of outer membrane barriers

Measurement of permeability

The measurement of permeation across the outer membrane has proved difficult because of the presence of two cell-envelope membranes. In addition, uptake is via diffusion rather than active uptake, which creates problems with measurement sensitivity at physiologically meaningful substrate concentrations. The first problem has been overcome by providing enzymes in the periplasm (between the outer and cytoplasmic membranes) to 'capture' substrates as they cross the outer membrane. The interaction of externally added substrates with these enzymes is limited by the rate of entry into the periplasm and, thus, the actual steady-state rate of substrate conversion to products is a measure of the rate of diffusion across the outer membrane. The most successful application of this general procedure has been performed with the β-lactams, using periplasmic β-lactamase as the capture enzyme. Methodological improvements have permitted measurement of the rate of uptake at concentrations close to the minimal inhibitory concentrations (MICs) of the β-lactams[14]. Uptake rates are usually expressed as a permeability coefficient (P in nm s^{-1}) according to Fick's law of diffusion: $V = P \times A(S_o - S_i)$, in which V is the rate of diffusion (in nmol mg $cells^{-1}$ s^{-1}), A is the area of the cell surface, per unit weight (in nm^{-2} mg $cells^{-1}$), through which the antibiotic is diffusing, and S_o and S_i are the substrate concentrations (in nmol ml^{-1}) outside and inside the outer membrane, respectively, such that their difference represents the concentration gradient. The actual measured permeability coefficients of selected antibiotics have been described[14–17]. To put these into perspective, the barrier effect in *E. coli* can be calculated based on the approximate number of porin molecules (2×10^5 $cell^{-1}$) and their limiting diffusion area at the most constricted part of the OmpF porin channel (0.77 nm^2) to give an area available for diffusion of 0.15 μm^2. As the surface area of an *E. coli* cell is ~3 μm^2, this means that only ~5% of this surface area is available for diffusion. In addition, the large size of β-lactams, compared with the above constriction zones of porins, and the fact that this creates frictional and intermolecular interactions between the β-lactam and the amino acids lining the porin channel (which are difficult to estimate but probably impede β-lactam diffusion at least 100-fold[2,18]) mean that the rates of diffusion of β-lactams and their consequent access to their cellular targets (penicillin-binding proteins at the outer surface of the cytoplasmic membrane) is probably decreased by more than 1000-fold by the presence of a semi-permeable outer membrane.

The measurements of uptake of different β-lactams have indicated certain general principles. First, individual bacteria can differ substantially in their permeability to β-lactams (see below). Second, the chemical nature of individual β-lactams can influence their permeation rates strongly. As the interior of porin channels usually contains excess negatively charged amino acids (almost all porins are acidic proteins), they favour the passage of zwitterionic β-lactams rather than negatively charged molecules[15,17]. Also, β-lactams that are small and have limited bulkiness would diffuse more rapidly through porin channels[16,18].

Reducing minimal inhibitory concentrations of antibiotics

Mutational alterations of the specific outer membrane macromolecules, use of polycations or divalent cation chelators, and genetically engineering bacteria for increased outer membrane porosity have been shown to increase outer membrane permeability and make bacteria more susceptible to antibiotics. A variety of types of mutations are known to increase outer membrane

permeability[19]. However, some of the mutations previously thought to increase outer membrane permeability are now thought to have alterations that reduce active efflux[4], although certain mutations that make cells more susceptible to antibiotics have defined outer membrane changes[19]. For example, LPS rough mutations, which remove large portions of the rough core sugars of LPS, seem to influence the stability of outer membranes (probably due to decreased negative charge, which reduces divalent cation binding) and increase the ability of hydrophobic antibiotics to partition into the membrane, leading to reduced MICs (Ref. 11). In bacteria with moderate outer membrane permeability, such as *E. coli*, only the more hydrophobic antibiotics are affected. However, in less-permeable bacteria, such as *P. aeruginosa*, even antibiotics that are generally considered to be hydrophilic can be affected[12]. A different type of LPS mutation, *tolA*-12 in *P. aeruginosa*, enhances the interaction of polycations with LPS and, thus, specifically increases susceptibility to aminoglycosides and other polycations[12]. Alterations in the Braun lipoprotein can also result in supersusceptibility to hydrophobic drugs[19].

The interaction of polycations with divalent cation binding sites on the surface LPS of Gram-negative bacteria causes permeabilization of the outer membrane[20]. This can result in enhanced uptake of a variety of antibiotics and a consequent reduction in MICs, as shown with polymyxin B nonapeptide and selected cationic antimicrobial peptides[21–23]. A similar effect is seen with chelators of divalent cations[20], and numerous classical studies with ethylene diaminetetraacetate (EDTA) attest to its ability to permeabilize the outer membrane and act synergistically with many antibiotics in several bacterial species.

We recently demonstrated the importance of the outer membrane barrier by increasing porosity using molecular genetic means[24]. An eight-amino-acid deletion in presumptive loop 5 of the specific porin OprD of *P. aeruginosa* increased the ion permeability of this channel by more than 20-fold. When cloned back into *P. aeruginosa* in a high-expression cassette, this OprDΔL5 porin led to a reduction in MICs by 8–16-fold for β-lactams, quinolones, tetracycline and chloramphenicol, all of which are presumed to cross the outer membrane via the porin pathway, but not to aminoglycosides and polymyxin B, which utilize the self-promoted uptake pathway.

Low outer membrane permeability

Intrinsic resistance to hydrophilic antibiotics

Bacteria that fall into the category of non-fermentors demonstrate high intrinsic resistance to all classes of antibiotics (see Ref. 25). Such bacteria include *P. aeruginosa*, *Burkholderia* (*Pseudomonas*) *cepacia*, *Stenotrophomonas* (*Xanthomonas*/*Pseudomonas*) *maltophilia* and *Acinetobacter baumannii* (*calcoaceticus*). Each of these species is deficient in the porin pathway and has an outer membrane permeability to β-lactams that is 1–5% of that of *E. coli*. Coupled with effective co-determinant resistance mechanisms, including an inducible β-lactamase and active efflux, this renders these bacteria resistant to most antibiotics and difficult to treat in the clinic. Effective antibiotics have been developed for the wild-type strains of these species. However, even for these antibiotics, the usual MIC is so high that a single minor mutational alteration is sufficient to raise the MIC to a level that makes the bacterium clinically untreatable with that antibiotic. For example, the most recent β-lactams (cefpirome and cefepime), which represent the fourth generation cephalosporins, have MICs for *P. aeruginosa* that are 60-fold higher than those for *E. coli*[25].

Intrinsic resistance to polycations

Most bacteria are susceptible to polycations, and aminoglycosides have been one of the more reliable drugs available to the clinician. However, it is well known that *B. cepacia* is highly resistant to the polycationic antibiotics, aminoglycosides and polymyxins. This results from the inability of the polycations to interact with the outer membrane, probably because the low phosphate and high arabinosamine content of *B. cepacia* LPS preclude polycation (and divalent cation) binding and consequent self-promoted uptake[12,25].

Porin-deficient mutants

Clinically derived, porin-deficient mutants of several Enterobacteriaceae resulting from β-lactam therapy have been described[1,12]. Although such mutants are likely to be uncommon, they may be important in conjunction with other resistance mechanisms. Laboratory-derived, porin-deficient mutants of *E. coli* are 2–16-fold more resistant to β-lactams and 2-4-fold more resistant to other antibiotics that use the porin uptake pathway than their parent strains. A more common porin deficiency is the loss of the carbapenem-specific porin OprD in *P. aeruginosa*[24], which occurs in as many as 50% of patients treated with the carbapenem β-lactam, imipenem.

Secondary (co-determinant) resistance mechanisms

Even in a poorly permeable bacterium such as *P. aeruginosa*, β-lactams will equilibrate across the outer membrane in as little as 10–100 s, whereas in *E. coli* the equilibration time is usually less than 1 s. Thus, low outer membrane permeability cannot result in resistance by itself. However, outer membrane permeability is critically important to antibiotic susceptibility because decreasing outer membrane permeability leads to antibiotic resistance, whereas increasing outer membrane permeability results in supersusceptibility. It is clear, therefore, that secondary (co-determinant) resistance mechanisms, which take advantage of the relatively slow movement of antibiotics into the periplasm, must exist. Two such co-determinant resistance mechanisms have been described: β-lactamases and active efflux (Fig. 2).

For the β-lactams, the production of periplasmic β-lactamase can result in resistance, even for those β-lactams that are poorly hydrolysed by this enzyme[15–17,26]. Intrinsic resistance to β-lactams usually involves synergy between restricted outer membrane permeability

and chromosomal class C cephalosporinases, which are induced by the β-lactams themselves. Mutational resistance to β-lactams, and especially clinical resistance, usually results from either plasmid-encoded β-lactamases or, in certain species, from derepression (*ampD* mutations) of chromosomal β-lactamases. In both cases, the influence of increased (periplasmic) β-lactamase expression is magnified by restricted passage of β-lactams through the outer membrane.

Recent studies have stressed the importance of active efflux in intrinsic and mutational resistance to antibiotics[3,4]. Thus, deletion by mutation of the most predominant active-efflux system in a given species can reduce MICs for hydrophobic antibiotics by up to 100-fold, and for amphipathic and even relatively hydrophilic antibiotics by 2–8-fold. Conversely, overexpression, through regulatory mutations of certain efflux systems, can result in resistance to a variety of antibiotics. However, these efflux systems are co-determinant resistance mechanisms because increasing outer membrane permeability by mutation, polycation treatment or porin overexpression can apparently overwhelm efflux and lead to similar MICs to those observed with mutants that have lost their predominant efflux pathway. The actual mechanistic details of active efflux are still being worked out, but the most important efflux systems in Gram-negative bacteria are the RND (resistance, nodulation and division) systems[3], which involve an energized, low-specificity cytoplasmic membrane pump, a periplasmic 'link' protein of unknown function and, at least in *P. aeruginosa*, an outer membrane protein that may function as a gated porin.

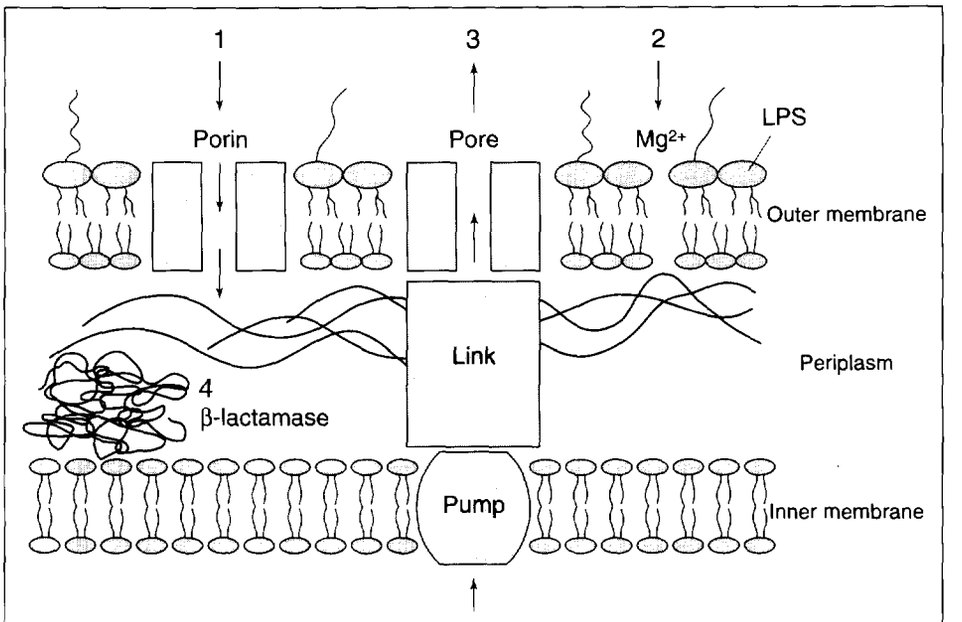

Fig. 2. Schematic diagram of the cell envelope showing the major outer membrane uptake systems and secondary (co-determinant) resistance mechanisms. The numbers refer to uptake mechanisms: (1) porin pathway for small hydrophilic molecules, and (2) self-promoted uptake pathway for small polycations; and secondary resistance mechanisms: (3) three-component antibiotic efflux system, and (4) periplasmic β-lactamase. LPS, lipopolysaccharide.

How to overcome the outer membrane barrier

The most intensive efforts in the manipulation of antibiotics to improve efficacy have been made with the β-lactams. Tens of thousands of β-lactam variants have been synthesized at several pharmaceutical companies in an attempt to improve their spectrum of activity. It now seems that the compounds with improved Gram-negative activity that arose from these attempts were actually countering secondary or co-determinant resistance mechanisms, specifically β-lactamases. An alternative approach that is specifically targeted at this resistance mechanism is the co-administration of a β-lactamase inhibitor and a β-lactam. Unfortunately, by redesigning these β-lactamases through subtle amino acid mutations in residues surrounding the active site, bacteria have mutated to circumvent and overcome both novel enzyme-resistant β-lactams and β-lactamase inhibitors.

As the existence of an outer membrane permeability barrier is a major factor in reducing antibiotic effectiveness, an alternative anti-resistance strategy would involve the co-administration of a compound designed to breach this barrier and an antibiotic. This type of strategy has been considered previously using EDTA, polymyxin B nonapeptide and ascorbate, all of which are outer membrane permeabilizers[20]. However, there is now an unequalled opportunity for molecular design of novel permeabilizers. Antimicrobial cationic peptides are ubiquitous in nature and represent an important, or even the predominant, method of killing invading microorganisms. We have demonstrated clearly that these peptides interact with the outer membrane to permeabilize it and are taken up by self-promoted uptake[22,23]. Based on this observation, peptides that overcome the outer membrane barrier and are synergistic with antibiotics against Gram-negative bacteria can be designed. The ability to produce such peptides recombinantly[22,23] can be manipulated to produce an enormous number of analogues. Thus, we feel that such

Questions for future research

- How does permeation of hydrophobic substances occur across the outer membrane?
- Is the subsequent interaction with efflux systems affected by the extent of hydrophobicity of molecules?
- In self-promoted uptake, how do polycations pass through the hydrophobic interior of the membrane?
- How do the presumed outer membrane pore proteins that are involved in efflux actually function?
- Can active efflux of molecules that act in the periplasm (e.g. β-lactams) occur, and how important is this relative to β-lactamase activity?

peptides or their mimetics have excellent potential as additives for enhancing antibiotic activity against Gram-negative bacteria.

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Coffee-table target

The Wellcome Trust Illustrated History of Tropical Diseases
edited by F.E.G. Cox

The Wellcome Trust, 1996.
£35.00 hbk (454 pages)
ISBN 1 869 83586 7

The Wellcome Trust Illustrated History of Tropical Diseases is a beautiful coffee-table book of the highest quality: it has glossy paper, is attractively laid out and has beautiful illustrations. If this was the aim of this book, then I would have nothing but praise for it. It covers what the authors claim to be the history of 41 tropical diseases and nutritional disorders from cholera to yaws, hepatitis to Burkitt's lymphoma, amoebiasis to malaria, schistosomiasis to hookworm and sickle-cell disease to scurvy.

But, unfortunately, the Editor, the various authors and The Wellcome Trust envisage this book as more than just an adornment of coffee tables; it is supposed to be a historical text. The Trust chose various clinicians and scientists – experts in the field of tropical diseases – to write the chapters, all of whom are alleged to have 'a sense of history' (p. 7). But whatever having a sense of history actually means, I would assume that, at the minimum, the claimants would have enjoyed reading and have read historical articles and books. In this case, that assumption is palpably false; the authors have obviously not read or digested any history because they all seem to believe history to be a sequential summary of famous and correct primary sources from the past. Not one of the chapters provides more than that and, as far as I can see, very few of them include any bibliographical references to any modern historical works on these diseases. Even more extraordinary, the Editor explains that smallpox has been omitted from the list of diseases because its history has been told so well elsewhere. Can the same claim not be made about yellow fever and cholera? Yet, the article on cholera refers to only one of these historical works[1] and none of the many articles concerning the history of yellow fever has been mentioned.

The reasons for publishing this book are not clear to me. Apart from providing an interesting addition to the coffee table, it pales in comparison with *The Cambridge World History of Human Diseases*, which was published only three years ago[2]. In fact, it is almost as if someone with a 'feel' for invertebrates has written a textbook in 1996 that follows the format of Borradaile *et al.* in their old and wonderful text of 1932 (Ref. 3). *The Wellcome Trust Illustrated History of Tropical Diseases* must surely be branded as a relic of the past even though, unlike the 1932 text, it is filled with impressive photographs and produced on the most expensive paper.

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