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Leading articles

Antibiotic uptake: unusual results for unusual molecules

By any criteria antibiotics are unusual molecules. Many are semi-synthetic or synthetic and thus are direct products of the pharmaceutical chemists' ingenuity. However even the so-called 'natural' antibiotics derived from micro-organisms have unusual features, and often bear little resemblance to natural bacterial substrates. In addition, they are usually so-called 'secondary' metabolites; end products of metabolism which are excreted by their producing cells rather than taken up. Thus it is perhaps not surprising that antibiotic uptake assays often demonstrate unusual results and that, with few exceptions, antibiotics usually do not utilize normal substrate uptake pathways.

To permit discussion of what is unusual, it is important to first describe what is meant by 'normal' pathways of substrate uptake. There are basically three classes of uptake pathways: simple diffusion, facilitated diffusion and active transport (Jain & Wagner, 1980). Simple diffusion can involve uptake directly through the membrane bilayer according to the concentration gradient of substrate across the membrane. Two factors decree the rate of uptake in this case, these being the concentration gradient of substrate and the partitioning coefficient (basically the hydrophobicity or ability of a substance to dissolve into the membrane interior). An example of this type of simple diffusion might be the uptake of water across the cytoplasmic membrane. Another type of simple diffusion would be the uptake of substrates through a non-specific water filled channel such as the general porin proteins of the Gram-negative bacterial outer membrane. This type of simple diffusion also responds to the concentration gradient of substrate, but in addition the size of the substrate relative to the channel opening and its charge relative to the charge of the amino acids lining the porin channel is important (Hancock, 1987*a*). Facilitated diffusion involves uptake through a channel or carrier which is specific for a given substrate due to its possession of a specific binding site. Uptake by such a route is substrate-specific and dependent on the concentration gradient of substrate across the membrane, although the rate of uptake is dependent on the equilibrium constant for binding of the substrate to the channel or carrier. There are no proven facilitated diffusion systems for substrates across the cytoplasmic membrane. Although glycerol transport has been reported to involve facilitated diffusion (Lin, 1978), the lack of demonstration of a specific channel protein and the high permeability of glycerol across lipid bilayers, makes this conclusion suspect. Nevertheless, several substrate-specific porins have been reported in the outer membranes of Gram-negative bacteria (Hancock, 1987*a*), including those which can mediate substrate-specific uptake of imipenem (Trias & Nikaido, 1990) or of cathechol β-lactams (Curtis *et al.*, 1988). Although both simple and facilitated diffusion systems cannot *per se* lead to concentrative uptake, their coupling to other uptake systems, such as those across the cytoplasmic membrane, or to metabolism, can result in maintenance of the substrate concentration gradient thereby producing higher intracellular concentrations of the substrate or its metabolite.

Active transport involves uptake across the energized cytoplasmic membrane. The expenditure of cellular energy in the form of either high energy phosphate bonds or the cytoplasmic membrane proton gradient provides the driving force for this concentrative uptake, whereby the final substrate concentration in the bacterial cytoplasm can exceed the external concentration by 50-fold or more. Active transport is usually highly substate specific and obeys Michaelis-Menten kinetics.

In the light of this information, we would like to consider the uptake of three classes of antibiotics, β-lactams, aminoglycosides and fluoroquinolones, all of which present several surprising features. β-Lactams are thought to have relatively easy access in Gram-positive bacteria to their target penicillin binding proteins which are situated on the external face of the cytoplasmic membrane. However in Gram-negative bacteria, they must cross the outer membrane permeability barrier (Hancock, 1987*b*). Some evidence has accumulated for the existence of non-porin pathways of uptake across the outer membrane, especially in certain outer membrane mutants.

However the predominant pathway is still considered to be through the water-filled channels of porin proteins. The biophysical and biochemical properties of these channels have been well studied in model membrane systems and thus the guiding principles for movement of molecules through these channels are rather well defined (Hancock, 1987*a*). Nevertheless two interesting and unusual results have recently come to light. The first of these issues involves the passage of 'third' and 'fourth' generation cephalosporins through porin channels. If molecular models of these molecules are built for example by using the molecular modelling program Alchemy (Tripos Associates Inc., St. Louis, USA), they are found to be ellipsoid with a long axis of 1·5 nm and diameter of 0·5–0·8 nm. The limiting diameter of the porin channels of *Escherichia coli*, such as OmpF porin, has been shown to be 1·16 nm in several model membrane systems (Hancock, 1987*a*). Thus it is obvious from a consideration of these dimensions alone that β-lactams must move in a highly oriented fashion (i.e. bullet-like) through porin channels. Consistent with this is the demonstration (Nikaido, Liu & Rosenberg, 1990; Bellido, Pechère & Hancock, 1991) that the positive charge of fourth generation cephalosporins, which is located towards the tip of these ellipsoid molecules, results in a substantial increase in rate of permeation through the porins of *Enterobacter cloacae*, when compared to the permeation of third generation cephalosporins which lack such a positive charge. It was concluded by us that the positive charge serves to orientate these molecules and permit their easier uptake through the presumably cation selective porin channels.

A second surprising finding relates to the broad spectrum carbapenem, imipenem. This β-lactam is highly stable to β-lactamases, has a rather low molecular weight and size, and like the fourth generation cephalosporins, is zwitterionic. These characteristics would seem to be ideal for optimal activity and uptake through porins. However despite its broad spectrum, imipenem does not generally have very low MICs for Gram-negative bacteria (Jones *et al.*, 1989). Indeed it is known that imipenem uses an alternative specific porin OprD in *Psudomonas aeruginosa* (Trias & Nikaido, 1990) and possibly also in *Enterobacter aerogenes* (Hopkins & Towner, 1990), whereas our own studies (Bellido, F. & R. E. W. Hancock, unpublished data) have shown that imipenem has a far lower rate of permeation in *E. cloacae* than cephaloridine or the fourth generation cephalosporins, cefpirome and cefepime, and appears to be unaffected by mutations influencing the porin uptake pathway for most β-lactams. These unusual findings suggest that imipenem (molecular weight 299) permeates very poorly through the porin channels that accommodate β-lactams of much higher molecular weight such as cefpirome (molecular weight 500). Several potential explanations occur to us. Since the positive charge of imipenem occurs at the end of a flexible side chain, it may be possible that this positive charge neutralizes the negatively charged carboxyl attached to the carbapenem ring, giving imipenem substantially different biophysical properties compared to the other zwitterionic β-lactams mentioned above, which have fixed positive changes. Alternatively imipenem might form head to tail dimers, thus presenting itself to the porin in a form that is too bulky to pass through the porin channel. It is also possible that the interaction between imipenem and Class I β-lactamase does not obey Michaelis-Menten kinetics under microbiologically or clinically relevant conditions. However our own recent studies would seem to provide evidence against this possibility.

Aminoglycoside uptake has been the subject of numerous papers and reviews (Hancock, 1981*a*, *b*; Bryan & Kwan, 1983; Nichols & Young, 1985; Taber *et al.*, 1987). Bryan & Kwan (1983) described aminoglycoside uptake in terms of three uptake phases: an initial binding phase, a slow but accelerating energy dependent uptake phase (EDP-I) and a rapid energy dependent uptake phase (EDP-II). This uptake system has several interesting and unusual features. Firstly the transition between EDP-I and EDP-II is coincident with the onset of killing (Hancock, 1981*a*, *b*) and is induced by aminoglycoside action, although there is no apparent specificity with respect to the inducing aminoglycoside. Secondly most cells have suffered a lethal event before completion of more than 25% of EDP-II (Hancock, 1981*b*). Thirdly the amount of aminoglycoside influences the timing of the onset of EDP-II, rather than the kinetics of uptake during EDP-II (Nicas & Hancock, 1980) suggesting that a critical level of aminoglycoside must be achieved intracellularly in order to trigger the EDP-II phase. Taken together, these data suggest that the EDP-II phase of aminoglyside uptake is not relevant to the lethal action of aminoglycosides since it occurs subsequently.

Aminoglycoside uptake also demonstrates

unusual energy dependent characteristics with a dual requirement for the electrical potential gradient component of the proton motive force and for a functioning electron transport chain (Bryan & Kwan, 1983). The former, oriented interior-negative, would seem to be the actual driving force for uptake of cationic aminoglycosides across the cytoplasmic membrane, whereas Bryan & Kwan (1983) have suggested that the electron transport requirement reflects a role for respiratory quinones as carriers. However there is no direct proof of a role for quinones or indeed other membrane components as carriers of aminoglycosides and it is difficult to envisage their specificity for aminoglycosides.

Most energy dependent transport systems undergo a process of exchange diffusion when excess unlabelled substrate is added to cells loaded with radiolabelled substrate i.e. intracellular radiolabelled substrate is exported as extracellular substrate is imported. This is not true for aminoglycosides as demonstrated by Nichols & Young (1985). This suggests that either aminoglycoside passage across the cytoplasmic membrane is kinetically irreversible or that aminoglycosides are irreversibly trapped inside the cytoplasm of bacterial cells. We favour the latter explanation, since it is known that aminoglycosides can precipitate DNA (Moskowitz, 1963) and such co-precipitation inside cells would effectively prevent exchange diffusion.

Aminoglycoside passage across the outer membranes of Gram-negative bacteria also employs an unusual non-porin pathway. In both *P. aeruginosa* (Hancock & Bell, 1988) and *E. coli* (Hancock *et al.*, 1991) aminoglycosides promote their own uptake across the outer membrane. Thus the bactericidal action of aminoglycosides could be correlated to the ability of the compounds to bind to the LPS and make permeable the outer membrane (Loh, Grant & Hancock, 1984; Rivera *et al.*, 1988; Jackson, Lolans & Daikos, 1990; Hancock *et al.*, 1991) as the initial step of self-promoted uptake.

The analysis of quinolone uptake is a major cause for concern due to inconsistencies both in *P. aeruginosa* and *E. coli* (Bedard *et al.*, 1989; Hooper *et al.*, 1989; Diver, Piddock & Wise, 1990; Fukuda *et al.*, 1990; Masecar, Celesk & Robillard, 1990). Without going into detail these include: the substantial backgrounds (zero time values which can be partly suppressed by increasingly vigorous washing); frequent lack of observed time kinetics; variable effects of carbonyl cyanide m-chlorophenylhydrazone (CCCP) and other inhibitors; differences in uptake conditions necessary to show transport defects in ciprofloxacin resistant mutants; lack of correlation between whole cell inhibitory concentrations in intact cells and MIC in *P. aeruginosa* compared to *E. coli*; inconsistencies between concentrations resulting in DNA gyrase inhibition and MIC in *E. coli* (Hooper *et al.*, 1989) suggesting concentrative uptake; the linearity of uptake levels as a function of ciprofloxacin concentration, that is more consistent with simple or facilitated diffusion; disparities between Mg^{2+} effects on MIC and lack of Mg^{2+} inhibition of uptake and the lack of correlation in *P. aeruginosa* compared to *E. coli* of MIC with measured rates of uptake. Detailed studies have been performed in *P. aeruginosa* by Bedard *et al.* (1989) and in *E. coli* by Diver *et al.* (1990). However, we do not feel that productive ciprofloxacin uptake (leading to internalization and target inhibition) has been definitely demonstrated or measured. Clearly this will await the isolation of mutants specifically influencing trans-cytoplasmic membrane uptake. Nevertheless it is difficult to see how hydrophilic molecules, like quinolones, could be taken up by simple diffusion across the cytoplasmic membrane, as suggested by some authors. In addition the contribution of an energy dependent low affinity export system for fluoroquinolones is somewhat unclear (Bedard *et al.*, 1989). One possible explanation for the observed disparities may be that quinolones cross the outer membrane by simple diffusion to form a pool of bound quinolone in the periplasm resistant to removal by washing. This pool could then feed a putative cytoplasmic membrane uptake system for quinolones.

The route taken by fluoroquinolones in crossing the outer membrane is also a point of controversy. It has been suggested that either OmpF porin (Hirai *et al.*, 1986) or self-promoted uptake (Chapman & Georgopapadakou, 1988) pathways, or another porin-independent route (Hirai *et al.*, 1986; Piddock & Wise, 1986) are used to cross the *E. coli* outer membrane. In *P. aeruginosa* it has been variously suggested that the general porin OprF (Piddock, Wijnands & Wise, 1987), the imipenem-specific porin OprD (Michea-Hamzehpour, Lucain & Pechere, 1991) or the iron regulated protein OprG (Chamberland *et al.*, 1989) mediate fluoroquinolone uptake. Thus there appear to be some difficulties in correlating variation in fluoroquinolone MICs with the appearance

and disappearance of outer membrane proteins. This may in part relate to the observation that antibiotics can select mutants with quite pleiotropic phenotypes (Gutmann *et al.*, 1988; Hooper, *et al.*, 1989; Fukuda *et al.*, 1990).

This short synopsis with three of the more commonly used classes of antibiotics demonstrates how much we have yet to learn about antibiotic uptake. Even for a system as well characterized as simple diffusion through porin channels, there are outstanding questions. We feel it will continue to prove difficult to explain unusual findings for antibiotic uptake assays on the basis of experiences with classical substrate uptake pathways.

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When is penicillin monotherapy the antibiotic treatment of choice?

Injectable and oral penicillin are to be found on the shelves of all hospital pharmacies, but how often is penicillin indicated as sole antimicrobial therapy? While penicillin has the advantages of narrow spectrum and low toxicity, many other agents are effective in conditions where penicillin is regarded as 'classical' therapy (Table). The indications for penicillin include specific infective syndromes such as infective endocarditis and also individual micro-organisms, for example, Lancefield group A β-haemolytic streptococci.

β-Haemolytic streptococci (Lancefield group A) infections of the pharynx and tonsils have been studied extensively, but, for infections at other sites data are limited. The clinical response of pharyngitis to oral or intramuscular penicillin is good; 96% of patients being apyrexial and 67% having resolution of pharyngeal pain within 48 h. All symptoms have usually resolved at 13–15 days follow-up (Pankey *et al.*, 1981; Stillerman, 1986). However, failure of bacteriological eradication may occur in between 2% and 38% of patients treated with penicillin (Rabinovitch *et al.*, 1973; Kaplan & Johnson, 1989). This is of potential importance as failure of eradication in patients with pharyngitis or tonsillitis may lead to recurrence, rheumatic fever and occasionally acute glomerulonephritis (Catanzaro, Rammelkamp & Chanovitz, 1958). Oral cephalosporins, such as cefaclor and cephalexin, are equivalent agents to penicillin for the treatment of first episodes of infection (Disney *et al.*, 1971; Stillerman, Isenberg & Moody, 1972; Rabinovich *et al.*, 1973; Stillerman, 1986). However, for patients with recurrent tonsillitis eradication was significantly better with co-amoxiclav than penicillin and, those treated with co-amoxiclav had significantly fewer episodes of recurrence than a similar group treated with penicillin (Brook, 1989). In an outbreak of group A streptococcal pharyngitis in a semi-closed community, those patients in whom the group A streptococci were not eradicated by penicillin were significantly more likely to be bacteriologically cured by co-amoxiclav than by a repeat course of penicillin (Kaplan & Johnson, 1989). In con-