Arch. Biol. Med. Exp. 13,233-245, 1980

Bacterial Cell Characteristics that Contribute to Antibiotic Resistance

Características de la célula bacteriana que contribuyen a la resistencia a antibióticos

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(Recibido el 23 de abril de 1980)

ZEMELMAN, R., MONDACA, M.A., NEIRA, A., ACEVEDO, M. Bacterial Cell Characteristics that Contribute to Antibiotic Resistance. (Características de la célula bacteriana que contribuyen a la resistencia a antibióticos). *Arch. Biol. Med. Exp. 13*:233-245, 1980

Bacterial resistance to antibiotics is a complex phenomenon which is based on different types of mechanisms, depending on several factors mainly related to the bacterial resistant strains. Enzymatic inactivation of antibiotics appears to be the most important and well studied of the resistance mechanisms, and enzymes inactivating Beta-lactams, aminoglycosides, and other antibiotics have been described. In most cases, the synthesis of these enzymes is codified by plasmids, which can be transferred to susceptible strains. However, mechanisms not based on the activity of specific enzymes seem to play also important roles in the resistance to antibiotics, as shown by the experimental evidence presented and discussed in this paper. Among these mechanisms, the lack of binding of antibiotics to specific cell receptors (especially at the cytoplasmic membrane level), and decreased penetration of the drugs into the cells have been demonstrated in resistant mutants isolated in the laboratory, as well as in naturally occurring strains isolated from infectious diseases.

The refractoriness that a great number of bacterial strains exhibit to antibiotics has been the subject of numerous investigations (1), and a strong stimulus for the search of new molecules with antibacterial activity against resistant strains. Most of the work has been oriented towards the study of resistance due to enzymatic modifications of antibiotic molecules. Special emphasis has been placed on the study of enzymes that are synthesized through the genetic information of plasmids (2). Comparatively, little research has been carried out on the mechanisms which do not depend on the activity of specific enzymes.

In this paper, we discuss some of the latest experimental evidence that relate bacterial structure and resistance levels to the most commonly used antibiotics. Results obtained in our Department are also included and discussed.

In order to get an adequate understanding of the resistance mechanisms developed by microorganisms to antibiotics, it is necessary to comprehend the interactions that take place between the antibiotic and the bacteria. As shown in Figure 1, several factors are involved in the problem of antibiotic activity. Some of them are related to the antibiotic itself, others to the microorganisms receiving the antibiotic, and some to the environment which sorrounds the microorganisms and/or the drug (3). The most important factors that depend on the antibiotic are: a) the bacteriostatic or bactericidal activity, b) antibacterial spectrum, c) the concentration achieved in the site of bacterial multiplication, d) the degree of binding to plasma or tissue proteins, and f) the degree of binding to specific protein receptors in the bacterial cell.

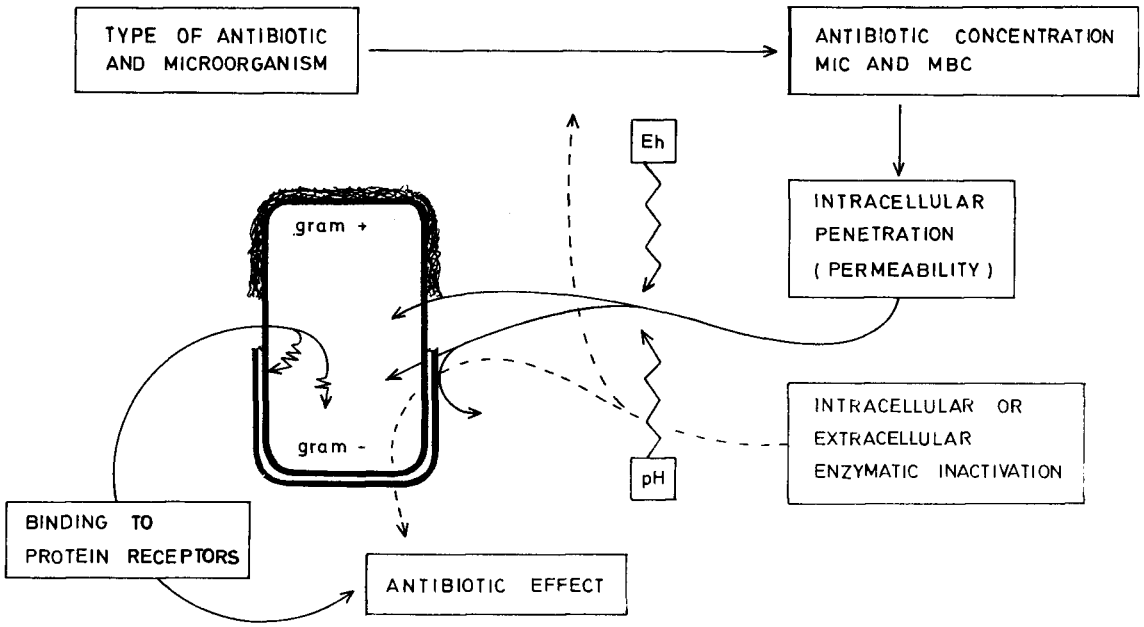

Fig. 1. Different factors that participate in the interaction of antibiotics and bacterial cells.

Among the factors that are dependent on the microorganism which is to be inhibited or killed, we have: a) the type of microorganism (Gram positive or Gram negative), b) the degree of multiplication at the tissue level, c) the physiological state of the cells being treated, d) the ability of the cells to synthesize specific enzymes which inactivate or modify the antibiotic molecule, either intra or extracellularly, and e) strain difference in components that behave as antibiotic receptors. The most important environmental factors are: a) pH at the site of bacterial multiplication, b) the presence of substances that are able to partially inactivate the antibiotic, and e) the redox potential. As an example of the latter, it has been shown that relative anaerobiosis decreases the activity of aminoglycoside antibiotics on strains of *Staphylococcus aureus* (4), and of Beta-lactam and aminoglycoside antibiotics on *Streptococcus faecalis* (5).

From the above it follows that the interaction of antibiotics with bacterial cells is not simple, and that the final effect of these on a given microorganism is the result of several factors that act in a chain of events that ends when the antibiotic reaches the target site where it must bind to specific receptors. Some of these have been identified as being part of important enzymes of bacterial metabolism (6).

Antibiotic activity is quantitatively assayed by their Minimal Inhibitory Concentrations (MIC) (7), their Minimal Bactericidal Concentration (MBC) (7), or their Minimal Antibiotic Concentrations (MAC) (8). However, the *in vitro* concentrations do not always parallel those required *in vivo* to obtain the same effects, due to some or all the factors that have been outlined above.

Different degrees of susceptibility towards a given antibiotic can be observed when separate strains of a bacterial species are subjected to it. Hence, the different strains can be grouped according to the concentration of antibiotic that is required to obtain effects such as alteration of morphology, growth inhibition, cell death, etc. It is possible, therefore, that for some strains it might be next to imposible to reach the effective antibiotic concentration when therapy is initiated, because the MIC of the antibiotic to be used could be higher than the level achieved when the antibiotic is administered. In this case, the strains are

classified as "resistant". For this reason, antibiotic concentration limits have been proposed in order to predict, as closely as possible, the outcome of antibiotic therapy *in vivo*. These relate the MIC or MBC with the plasma or tissue levels which are pharmacologically achievable (7). In other words, every bacterial strain is susceptible to a given antibiotic concentration and, at the same time, is resistant to lower concentrations of the same drug. However, the practical implications of the routine denominations of "susceptible" or "resistant" must necessarily take into account the wide range of factors described above.

THE MECHANISMS OF RESISTANCE TO ANTIBIOTICS IN BACTERIA

Benveniste and Davies (1) have suggested that bacterial resistance mechanisms to antibiotics can be classified in two broad categories:

1. Those based on chemical modifications of the antibiotic produced by bacterial enzymes that lead to partial or complete loss of antibacterial activity, and
2. Mechanisms based on structural alterations of cellular components that result in inhibition of antibiotic binding to specific receptors at the target site, or in a decreased penetrability of the antibiotic into the cell. These mechanisms are refered to as "intrinsic resistance".

1. *Antibiotic inactivating enzymes*

The mechanisms based on enzymatic modifications of the antibiotic are, by far, most frequent. They are also, in most cases, the phenotypic expression of extrachromosomal inheritance codified by plasmidic DNA (2). The best known enzymes of this kind are the Beta-lactamases (9), which hydrolyze penicillins and cephalosporins, and the aminoglycoside inactivating enzymes (2), on which valuable information has been published in the past few years. Both types of enzymes are produced by Gram positive and Gram negative bacteria, but when synthesized by the latter, they are concentrated in the periplasmic space that exists between the outer and the cytoplasmic membranes of these microorganisms (10). Therefore, Beta-lactamases and aminoglycoside inactivating enzymes of Gram negative bacteria are intracellular enzymes that exert their activity when their substrates reach the periplasmic space. On the other hand, the enzymes produced by Gram positive bacteria (*Staphylococcus* sp., *Bacillus* sp., etc.) are leaked to the environment and the antibiotics are inactivated extracellularly. Another important characteristic that differentiates Beta-lactamases synthesized by Gram positive microorganisms from those produced by Gram negative bacteria is their inducibility. Other antibiotic that can also be enzimatically inactivated is chloramphenicol by chloramphenicol-acetyltransferase produced by bacterial strains resistant to this antibiotic (11)Beta-lactamases have been intensively studied in the last decade. An important outcome of this research has been the production, by the pharmaceutical industry, of several Beta-lactam antibiotics which are not susceptible to the activity of Beta-lactamases produced by *Staphylococcus* sp. An important example of the above are the semisynthetic isoxazolyl penicillins (12), which have greatly advanced the therapy of staphylococcal disease. In contrast, research on antibiotics with activity against Beta-lactamase-producing Gram negative microorganisms has been less productive. A significant number of broad-spectrum penicillins have been described which have important pharmacological properties, but that are still susceptible to the hydrolytic activity of these enzymes. One of the last of these penicillins is amoxycillin (12). The specific inhibition of Gram negative Beta-lactamases has been another field of active research, since the activity of these enzymes appears to be the most important mechanism of resistance in these microorganisms. Natural as well as semisynthetic products have been obtained, i.e. clavulanic acid (13), and olivanic acids (14), that, when used in association with hydrolyzable penicillins (i.e. ampicillin, amoxycillin, etc.) have shown important drops in the resistance levels of various microorganisms (15). The use of clavulanic acid plus amoxycillin in the treatment of

urinary tract infections due to Beta-lactamase producing microorganisms has been recently reported as successful (16). Some new molecules have been obtained which share both effective antibacterial activity (which is low in clavulanic and olivnic acids) as well as inhibition of Beta-lactamase activity. Such is the case of thienamycin (17) which shows adequate antibacterial activity, even upon strains of *Pseudomonas aeruginosa*.

The action of Beta-lactamases on penicillin and cephalosporin molecules is well known. The reaction products are shown in Figure 2. It can be seen that penicillins and cephalosporins share a Beta-lactam ring which is opened by these enzymes with formation of the corresponding non-active penicilloic and cephalosporinoic acids of different stabilities. Although different types of Beta-lactamases have been described (9,

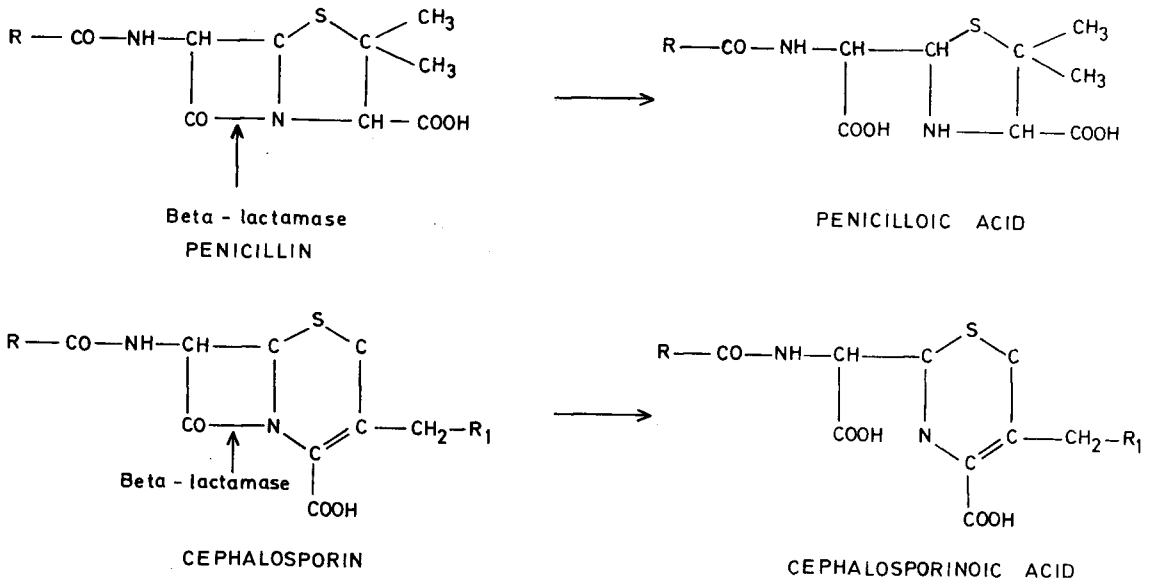

Fig. 2. Activity of Beta-lactamases upon penicillins and cephalosporin molecules, showing the target sites in the 6-aminopenicillanic and 7-aminocephalosporanic acids.

18), their activity is the same, since all penicillins share the common nucleus 6-aminopenicillanic acid, and all cephalosporins contain the 7-amino-cephalosporanic acid nucleus.

Aminoglycoside inactivating enzymes share a completely different mechanism of action. The chemical structure of this wide and complex family of antibiotics contains an aminocyclitol (streptidine or deoxistreptamine) substituted with aminosugars, and both types of molecules possess primary amino and hydroxyl groups which are susceptible to the attack by inactivating enzymes (19). Several such enzymes are now known and they have been grouped according to the chemical reaction which they catalyze (Fig. 3). Thus, aminoglycoside acetyltransferases (AcT), aminoglycoside phosphoryltransferases (AphT), and aminoglycoside adenyliltransferases (AdT) have been described in both Gram positive and Gram negative microorganisms (2). As a consequence of their enzymatic activity, important primary amino and hydroxyl groups of the antibiotic molecule are substituted (acetylated, phosphorylated or adenylilated), and, as has been suggested, the penetration of the modified antibiotics into the cells is inhibited (1). Loss of antibacterial activity is not necessarily due to

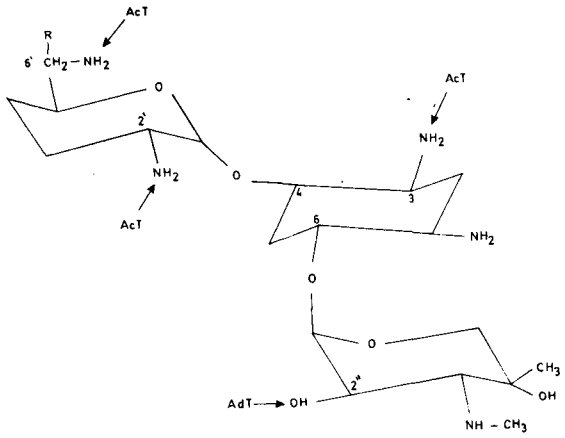

Fig. 3. Chemical structure of a gentamicin molecule showing target sites of acetyltransferases (AcT) and adenyliltransferase (AdT) upon primary amino and hydroxyl groups, respectively.

the chemical modification which takes place, but could be due to the inaccesibility of the modified molecule to its target site. At the present time, new enzymes of these types are being reported (20).

Although significant advances have been made in the study of the aminoglycoside inactivating enzymes in the past few years, no specific inhibitors have yet been reported. Hence, this is a field of research that can be rewarding, because the isolation of such natural inhibitors could lead to the restoration of the antibacterial activity of antibiotics that are now usually inactivated by resistant strains isolated from clinical specimens. However, semisynthetic antibiotics have been obtained that have been found to be resistant to most of the enzymes already mentioned, and which show important antibacterial activity against resistant strains to the naturally produced aminoglycosides. Such is the case of amikacin and dideoxykanamycin B (21), which exhibit a low frequency of resistant strains and are in wide use today.

2. *Intrinsic resistance*

As previously provided out, intrinsic resistance refers to the mechanisms which are not due to the hydrolytic activity of inactivating enzymes on the antibiotic molecules. The role of these naturally occurring mechanisms in pathogens is not completely understood, but the available evidence suggests that they depend on basic alterations of the bacterial structure. The possibility that these mechanisms could also play a role in bacteria that produce inactivating enzymes can not be ruled out.

The final consequence of the bacterial resistance mechanisms is the increase in the MICs and the MBCs of the antibiotics. A point is reached when these become higher than the levels which can be achieved in the organism through routine therapy. When this happens, bacterial multiplication continues unhindered, and the corresponding clinical signs are manifested as usual. Three types of intrinsic mechanisms of resistance have been described: a) decreased binding of the antibiotic to bacterial receptors, b) decreased penetration of antibiotics into bacterial cells, and c) impermeability at the cytoplasmic membrane level.

DECREASED BINDING OF THE ANTIBIOTIC TO BACTERIAL RECEPTORS

Research on resistance to Beta-lactam antibiotics has led to the identification of several protein components of the bacterial cytoplasmic membrane, which have been shown to play important roles in cellular metabolism and that bind Beta-lactam antibiotics. In fact, the effect of these antibiotics upon susceptible cells is now explained through their binding to one or more of these protein receptors. Beta-lactam binding components have been reported in *Escherichia coli, P. aeruginosa, Neisseria gonorrhoeae,* among others (22). In *E. coli* at least six proteins have been identified and are designated as proteins 1, 2, 3, 4, 5 and 6 (23). Some of these have been now resolved into two proteins. Some important characteristics of bacteria, such as morphology, elongation, cell division, etc., appear to be dependent on the activity of some of these proteins as shown in Fig. 4 (24). Matsuashi *et al.* (25) have found that protein 5 is associated with the activity of d-alanine carboxypeptidase in *E. coli* K-12, whereas Nakagawa *et al.* (26) have isolated

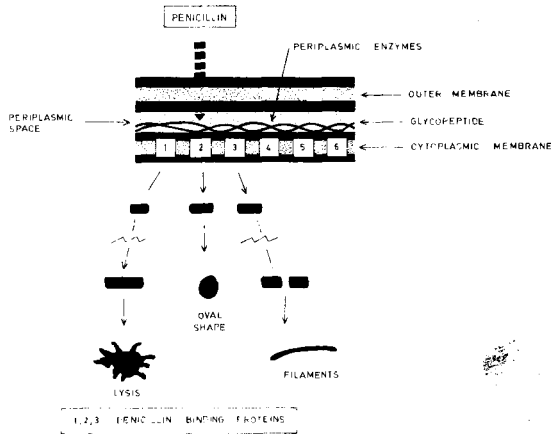

Fig. 4. Schematic structure of the envelope of *Escherichia coli* showing protein binding components for penicillin in the cytoplasmic membrane and the effect upon cell morphology after antibiotic action.

protein 1Bs (being part of the protein 1 complex) from *E. coli* cytoplasmic membranes and showed that this protein exhibits both peptiglycan polymerase and peptidoglycan transpeptidase activities. Therefore, on the basis of these results, penicillin activity can be explained in terms of the inhibition of specific enzymes of the peptidoglycan biosynthesis which are located on the cytoplasmic membrane.

Not all Beta-lactam antibiotics bind preferentially to the same protein receptors. Hence, the similarity of morphological changes induced by different Beta-lactam antibiotics suggest that they bind primarily to the same protein component in this case.

According to the literature, resistance to Beta-lactam antibiotics can also be explained by their decreased binding to cytoplasmic membrane protein receptors. The following reports support this hypothesis. Spratt (27) has shown that an *E. coli* mutant, defficient in protein 2, was resistant to mecillinam (a novel Beta-lactam antibiotic which is known to bind to protein 2 in *E. coli*). In the susceptible wild strain, the effect of mecillinam is observed by the formation of osmotically stable round cells, that eventually lyse (Fig. 4). This altered morphology is not observed in the protein 2-defficient mutant. These results suggest that the rod shape of *E. coli* is dependent on the activity of protein 2 and that resistance to mecillinam is related to the lack of binding of the antibiotic to the protein receptor. Similar results were obtained by Buchanan and Strominger (6) in a penicillin-resistant mutant of *Bacillus subtilis* defficient in protein V (which binds benzylpenicillin in this microorganism). Rodríguez and Saz (28) studied some penicillin-resistant strains of *N. gonorrhoeae* isolated from clinical cases of gonorrhoea and found decreased binding of benzylpenicillin to the cells envelope of these strains, in absence of Beta-lactamase production.

Lastly, Curtis *et al.* (29) have studied the degree of binding of various Beta-lactam antibiotics (penicillins and cephalosporins) to the protein receptors of a wild strain of *E. coli* K-12. Then, they compared the antibacterial activity of the antibiotic against that strain and against an isogenic permeable mutant (in which antibiotics activity is dependent only on the degree of binding to target site). They reported that the affinities of the compounds for their primary lethal protein binding targets showed close agreement with their antibacterial activities against the permeability mutants. These results emphasize the importance of Beta-lactam antibiotic binding to specific protein components of the cytoplasmic membrane and antibiosis. In addition, it is clear that a lack of binding to these proteins brings about resistance to the antibiotics. The lack of binding, as the cause of resistance, can also occur naturally, as demonstrated by Rodríguez and Saz (28).

Aminoglycoside antibiotics have also been shown to bind to specific proteins in order to exert their antibacterial activity (30). Streptomycin, one of the best studied drugs of this family, binds to protein S12 of the 30S ribosomal subunit in *E. coli* (32). There is evidence, however, that it also binds to other proteins of the same subunit (32). This binding results in an apparent misreading of the genetic information during protein synthesis. Aminoacyl-tRNA binding to the A site of the ribosomal subunit is also inhibited (1). Kanamycin and spectinomycin (properly an aminocyclitol antibiotic) bind to protein S6 and S5, respectively (1). Both causes

misreading during protein synthesis. In the case of gentamicin, one of the most widely used aminoglycosides, recent experimental evidence suggests a biphasic mode of action, which is dependent on the antibiotic concentration (33). It has been proposed that the antibiotic binds to two different sites on the ribosome, one of which is responsible for peptide chain elongation, and the other for reading the mRNA information. Tobramycin, an antipseudomonal aminoglycoside, has been reported to bind to two sites on the 50S ribosomal subunit (34). At low concentrations, protein synthesis is inhibited *per se*, whereas at higher concentrations misreading is produced.

Other antibiotics not belonging to the aminoglycoside family also bind to specific proteins. Among them, rifampicin binds to the Beta subunit of the DNA-dependent RNA polymerase in susceptible cells of *E. coli* (35) and *B. subtilis* (36). Erythromycin to proteins of the 50S ribosomal subunit (37), and thiostrepton to the protein BM-L11 of *Bacillus megaterium* and to protein L11 of *E. coli* (38). These latter two proteins exhibit immunological cross-reactivity and play important roles in initiation, elongation, and termination of peptide synthesis.

The non-enzymatic resistance to streptomycin has been connected with structural alterations of the S12 protein produced as a consequence of mutational events at the chromosomal level (39). In fact, Funatsu and Wittmann (31) have studied the aminoacid composition of the S12 protein isolated from wild streptomycin-susceptible strains of *E. coli,* as well as from resistant mutants. They found that the S12 protein from the resistant strains did not bind streptomycin, and that a single aminoacid substitution had taken place in the polypeptide chain. They showed that in three different resistant mutants, a lysine residue had been replaced by asparragine, threonine or arginine, and suggested that these alterations were the cause of the binding inhibition. Zimmermann *et al.* (40) studied the translational behaviour of 30S ribosomal subunits obtained from strains of *Streptococcus faecalis* which proved to be streptomycin-resistant. Misreading occurred when these subunits were used in an *in vitro* asay system for protein synthesis. These results indicate that mutational events which lead to altered ribosomal proteins can bring about lack of binding and, as a consequence of this, streptomycin resistance in the absence of aminoglycoside inactivating enzymes. Moreover, this phenomenon can occur naturally, probably because of the selection of resistant mutants when streptomycin selective pressure is exerted in the environment.

Cundliffe *et al.* (38) showed that a thiostrepton-resistant mutant of *B. megaterium* was totally devoid of the BM-L11 protein of the 50S ribosomal subunit, and the ribosomes derived from these strains were less sensitive to the action of the antibiotic than the wild type strain. In addition, when the missing protein was restored (BM-L11), wild type levels of activity in protein synthesis and suceptibility to the antibiotic was also restored. These experiments clearly indicate that antibiotic binding to one specific protein in the ribosomes conditions antibacterial activity, and that the lack of binding brings about resistance.

Resistant mutants to rifampicin are frequently isolated from susceptible strains (41). Resistance in this case seems to be due to the synthesis of altered Beta subunits of the DNA-dependent RNA polymerase (1), which are thus incapable of binding the antibiotic, and RNA synthesis can proceed without interference. Pestka *et al.* (37) demonstrated a relation between the binding of erythromycin and some of its analogues to the 50S subunit from *E. coli* and antibiotic activity. Low binding was observed when antibiotic activity was defficient. However, it sems that a lack of erythromycin binding to protein receptors is not always the cause of resistance. In fact, in strains of *S. aureus* erythromycin resistance is due to an alteration of messenger RNA itself, and not to an alteration of the ribosomal protein content (1).

Another interesting example of non-enzymatic resistance to antibiotics is that of polymyxin. Profound alterations of the outer and cytoplasmic membranes are produced when cells of *Salmonella typhimurium* are exposed to high concentrations of this antibiotic (42). Membrane permeability is so

altered that the resulting leakage of important metabolites to the extracellular environment result in cell death (43). Vaara *et al.* (44) have shown that polymyxin-resistant mutants of *S. typhimurium* present decreased binding of the antibiotic to the cell envelope, although no specific binding proteins have yet been identified. These authors also proved that lipopolysaccharide (LPS), located in the outer membrane, is the most important receptor for the antibiotic in this microorganism. Polymixin did not bind to the LPS from resistant mutants suggesting that, in this particular case, a carbohydrate is incriminated in antibiotic binding. However, the fact that the antibiotic produces alteration of the cytoplasmic membrane, in which no LPS has been demonstrated, indicates that it probably also binds to it.

All the evidence that has been presented and discussed allow us to conclude that antibiotic binding to specific receptors in the bacterial cell is a *sine qua non* condition for antibacterial activity, and that decreased binding leads to a parallel decrease in susceptibility levels.

DECREASED PENETRATION OF ANTIBIOTICS INTO BACTERIAL CELL

Decreased penetration of antibiotics has also been associated with antibiotic resistance in Gram negative bacteria (45). In this type of mechanism, the outer membrane has been primarily incriminated (46, 47), since this structure of the cells envelope has been shown to participate in some way in the penetrability of several molecules (antibiotic and non-antibiotic molecules) into the periplasmic space. In other cases however, the cytoplasmic membrane actively participates in the impermeability to antibiotics, as will be evidenced later. The cell envelope structural differences between Gram positive and Gram negative bacteria has afforded a satisfactory explanation to the higher refractoriness usually exhibited by Gram negative microorganisms to several antibiotics.

The most intensively studied antibiotics in relation to cell impermeability are the Beta-lactams, and some of the latest and convincing evidence in this respect will be analyzed. Moore *et al.* (48) compared the effect of benzylpenicillin, ampicillin, and carbenicillin upon the transpeptidation reaction during peptidoglycan biosynthesis in toluene-treated *E. coli* and *P. aeruginosa*. This treatment makes cells permeable to the entrance of several molecules. The susceptibility of the reaction to inhibition by the antibiotics in every strain was then compared with the antibiotic concentrations required to inhibit growth of microorganisms. The hypothesis was that the higher resistance of *P. aeruginosa* was due to a higher resistance of the transpeptidase in this species. Surprisingly, the transpeptidation reaction in *P. aeruginosa* was found to be more susceptible to inhibition by all the antibiotics tested than the same reaction in *E. coli*. The authors suggest that a lower penetrability of the antibiotics into *P. aeruginosa* cells was probably responsible for the observed results. Mirelman and Nuchamowitz (49) arrived at similar conclusion when studying the effect of several Beta-lactam antibiotics on the activity of *E. coli* and *P. aeruginosa* carboxypeptidases and transpeptidases. In these experiments, cefalexin was found to be active against *E. coli* and inactive against *P. aeruginosa*, whereas cefsulodin (a novel cephalosporin with antipseudomonal activity) was active upon microorganisms. However, in a cell-free system, transpeptidases and carboxypeptidases were found to be susceptible to inhibition by cefalexin an cefsulodin. The results led the authors to conclude that the differences found between the action of cefalexin and cefsulodin on these to different assays (intact cells and cell-free systems) could be attributed to a lack of penetration of cefalexin into *P. aeruginosa* cells. As will be discussed later, this apparent cell impermeability seems to be in some way selective, since antibiotics other than Beta-lactams appear to enter some strains of *P. aeruginosa* without difficulty.

A further understanding of the role played by cell impermeability in antibiotic resistance has been obtained from binding studies of Beta-lactam antibiotics to specific membrane proteins. Curtis *et al.* (50) investigated the interaction of several penicillins and

cephalosporins with purified cytoplasmic membranes, with the complete cell envelopes (both outer and inner membranes), and with intact cells of a particular strain of *E. coli*, and with a hyperpermeable mutant. They showed that these antibiotics were effectively bound to protein receptors of purified cytoplasmic membranes, but no binding was observed in intact cells submitted to the action of the antibiotic. In addition, the cells were resistant to the drug assayed. Both effective binding and antibiotic activity were demonstrated when the antibiotics were assayed upon the hyperpermeable mutant of the same strain of *E. coli*. The outer membrane did not interfere the binding of the antibiotics to the inner membrane according to the results obtained when complete envelopes were used. The authors concluded that a satisfactory antibiotic activity could not be observed in intact cells because of the impermeability exhibited by the outer membrane. Thus, the outer membrane seems to exert its impermeability only when the cell integrity has not been altered.

The impermeability of Beta-lactamase producing Gram negative bacteria has been expressed through the Cripticity Factor (CF), which is calculated by relating the enzymatic activity of cell-free preparations (by disruption with the French press or ultrasonically), and the activity of intact cells (provided there is no leakage of the enzyme to the environment) (45). This calculation is based on the fact that antibiotic inactivation can only take place if the drug can enter the cell. Consequently, a CF close to 1 indicates permeability (or ease of entrance), and a high CF indicates impermeability. Unfortunately, the CF can only be calculated if enzymatic activity can be measured by the methods commonly used. Some strains do produce Beta-lactamase in such minute amounts that its determination is quite difficult to assess. Richmond *et al.* (51) have recently reported a method for determining the CF when Beta-lactamase-resistant penicillins are studied. In this method, the susceptibility of a strain of *E. coli* is compared with that of two mutants with varying permeability (hyper-permeable mutants). The differences in the MICs, between the wild and mutant strains give an indication of the relative penetration of the antibiotics into the cells.

The frequency of resistant strains of *E. coli*, *Salmonella* sp. and *Proteus* sp., routinely isolated from clinical specimens, has been compared with the average CF of every one of these strains. A direct relationship between resistance and impermeability has been observed (52), suggesting that a higher impermeability (as shown by higher CFs) might be involved in the resistance problem. A more refined method for determining impermeability has been proposed by Zimmermann and Rosselet (53), in which kinetic parameters are determined in order to avoid the problem of enzyme non-saturation in the periplasmic space when Beta-lactamases are assayed in intact cells. Even though the results seem to differ quantitatively with the former methods, they confirm the existence of a permeability barrier in the envelope of Gram negative bacteria.

The permeability role of the outer membrane has been further emphasized by results of experiments in which the entrance of the antibiotic has been chemically modified. For example, EDTA-treated cells of *E. coli* exhibit a higher penetration of Beta-lactam antibiotics, aminoglycosides, and other antibiotics, than non-treated cells (54). A corresponding lower MIC for every antibiotic was also observed, in addition to a relationship between the molecular weight of the antibiotic and the ease of entrance into the cell.

This type of approach was used in our laboratory to study the effect of gentamicin on a susceptible (*E. coli* K-12) and on a gentamicin resistant strain of *E. coli* (*E. coli* UCC35), in the presence and absence of subinhibitory concentrations of EDTA. Table 1 shows that the chelating agent decreases the MICs of gentamicin upon both strains. It is known that EDTA binds to the LPS in Gram negative bacteria, thus increasing their permeability (46). Therefore, the increased susceptibility can be attributed to a higher penetrability of gentamicin into the cells under study. In another type of experiment, also conducted in our laboratory, the inactivation of gentamicin, monitored by a microbiological method (55) was studied using both intact and

disrupted cells (disruption performed in the French press) as enzyme source (Table 2). The strains used were: a) two gentamicin-resistant strains of *E. coli* (UCC47 and UCC35), b) four exconjugant strains of *E. coli* K-12 obtained by conjugation of *E. coli* UCC47 (two exconjugants) and *E. coli* UCC35 (two exconjugants) with *E. coli* K-2, and c) one strain of gentamicin-resistant *P. aeruginosa* (UCPA271). The assay of gentamicin was also performed with *E. coli* UCC47 and *E. coli* UCC35 after treatment with toluene, whose

TABLE 1

Effect of a subinhibitory concentration of EDTA upon the activity of gentamicin in *Escherichia coli*

	MIC of gentamicin	
Microorganisms	*No EDTA*	*with EDTA*
E. coli K-12	1*	0.125
E. coli UCC35	512	64

*mcg/ml.

TABLE 2

Inactivation of gentamicin by cells of *Escherichia coli* and *Pseudomonas aeruginosa* either intact, disrupted in the French press or treated with toluene

Microorganisms	*MIC**	*Inactivation of gentamicin (%) by cells*		
		Intact	*Disrupted*	*Toluene treated*
E. coli UCC47	*1024*	*0*	*100*	*100*
E. coli UCC35	*1024*	*0*	*63.8*	*52.5*
*E. coli K-12 + UCC47-A***	*256*	*0*	*12.5*	
E. coli K-12 + UCC47-B	*256*	*0*	*51.3*	
E. coli K-12 + UCC35-A	*256*	*0*	*21.0*	
E. coli K-12 + UCC35-B	*256*	*0*	*23.1*	
P. aeruginosa UCPA271	*256*	*100*	*100*	

**Minimal Inhibitory Concentration (mcg/ml).*

***Exconjugant strains obtained by conjugation of E. coli* UCC47 and UCC35 and *E. coli* K-12.*

effect on membrane is known (56). As shown, only *P. aeruginosa* UCPA271 inactivated gentamicin when intac cells were used. When disrupted cells were assayed, all strains could inactivate gentamicin, although the exconjugants exhibited the lowest degree of antibiotic inactivation. The inctivation of gentamicin was also demonstrated when intact cells of *E. coli* UCC47 and UCC35 were assayed after treatment with toluene. Another finding was that MICs did not parallel gentamicin inactivation. These results indicate that in the wild and exconjugant strains of *E. coli* lack of penetration of gentamicin avoided inactivation, since solubilization of the periplasmic content by cell disruption allowed gentamicin inactivation in the assays, and a similar effect was obtained by increasing membrane permeability with toluene. *P. aeruginosa* UCPA271 was permeable, since gentamicin inactivation was similar when using intact or disrupted cells. In other experiments we have found strains of *P. aeruginosa* which exhibit impermeability to gentamicin. Hence the permeability observed in *P. aeruginosa* UCPA271 does not seem to be a general characteristic of resistant *P. aeruginosa*. The fact that the degree of gentamicin inactivation did not parallel the MICs is probably an indication that resistance can be due to both inactivation of gentamicin and cell impermeability.

The transfer of the ability to inactivate

gentamicin into *E. coli* K-12 demonstrates the presence of resistance transfer factors which codify the synthesis of the aminoglycoside-inactivating enzymes. A CF for expressing impermeability could not be calculated, since no inactivation of gentamicin was produced by intact cells of *E. coli*. In the case of *P. aeruginosa* UCPA271, CF would be equivalent to 1 (100% inactivation by intact or disrupted cells).

Outer membrane impermeability has been correlated with several parameters, the most important being the molecular weight and hydrophobicity of the antibiotic molecule (57). The presence of LPS also affects antibiotic penetration, since penetration has been shown to be higher in LPS-defficient mutants (46). The influence of the lipid composition of the cell has also been studied in relation to permeability, but no conclusive results have yet been obtained (58). More rewarding results have been obtained with research on the protein content of the outer membrane. In fact, several proteins have been identified (59) which appear to play a role of "channels" that allow the entrance of some molecules into the cell. For this reason, they are generally referred to as "porins" (60). Nikaido *et al.* (61) have studied the susceptibility of *S. typhimurium* and a porin-defficient mutant for cephaloridine (a cephalosporin antibiotic which easily penetrates the outer membrane of Gram negative bacteria), and demonstrated an impressive decrease in susceptibility in the mutant strain which lacked the porin. Presumably this protein is the one that participates in antibiotic entrance into *S. typhimurium* cells. To our knowledge, no natural resistant strains which lack some of these porins and are resistant to antibiotics have been yet isolated.

Hancock *et al.* (62) have isolated a protein that behaves as porin in a strain of *P. aeruginosa* (PAO1). This protein seems to be responsible for the diffusion of molecules with molecular weights up to 6000. In *E. coli*, this exclusion limit has been calculated to be approximately 600 (57). If the results presented in Table 2 are interpreted on the basis of this information, gentamicin inactivation by cells of *P. aeruginosa* UCP271 can be explained, since the antibiotic has a molecular weight far below the exclusion limit reported by Hancock *et al.* (62). The difference of the entrance of gentamicin in cells of *E. coli* and *P. aeruginosa* could also be a reflection of their different outer membrane protein compositions.

IMPERMEABILITY AT THE CYTOPLASMIC MEMBRANE LEVEL

Several lines of experimental evidence have stressed the role of the inner membrane (or cytoplasmic membrane) in antibiotic resistance. For example, the resistance exhibited by strains of *S. aureus* to tetracyclines is originated by an alteration of the transport mechanisms at the cytoplasmic membrane (1). Somopolinsky *et al.* (63) demonstrated that a tetracycline-resistant strain of *S. aureus* actively accumulated the antibiotic but at a lower rate than that found in a susceptible strain. The differential uptake was independent of the external antibiotic concentration. Similar conclusions have been obtained by investigating tetracycline resistance in *E. coli* (64). These results indicates that, in Gram negative bacteria, resistance to some antibiotics can be due to either the outer or the inner membranes, thus emphasizing the versatility of these microorganisms to survive in adverse environments. The participation of the cytoplasmic membrane in antibiotic resistance is also exemplified in the case of aminoglycoside antibiotics which are being inactivated by periplasmic enzymes. In fact, Davies *et al.* (2) have suggested that the lack of activity of these antibiotics after being acted upon by periplasmic enzymes is due mainly to a failure in the penetration rather than to a loss of antibacterial activity (65). The supposition is made that active transport mechanisms for aminoglycosides are present in the cytoplasmic membrane, and that the enzymatically modified antibiotics are not transported. Phosphomycin is another antibiotic for which active transport mechanisms have been reported (66), and it is rather suggestive that, when phosphomycin-resistant mutants are isolated,

some of the mutants are also resistant to aminoglycosides (67).

The failure of chemically modified, but otherwise active, aminoglycosides to enter the cells is a point that needs further clarification. In our laboratory, we have found that when gentamicin-resistant strains are incubated with low concentrations of this antibiotic, either one of two results is obtained: a) the antibiotic is fully inactivated or b) it remains partially or totally active in the supernatant. If this supernatant is now assayed upon several gentamicin-susceptible strains, no activity has been found if the result was that of a), and activity is determined if result was that . This is an indication that perhaps resistance to gentamicin in aminoglycoside-inactivating strains is due mainly to a lack of activity of the antibiotic. If the lack of entrance is the cause of the resistance, the supernatant obtained from a) would have been active at least for some species.

The results which we have commented in this paper stress the importance of several non-enzymatic factors which are responsible for the resistance of bacteria to antibiotics. These factors probably do not work at the same level in every bacterial species, but they appear to be present in strains which produce inactivating enzymes, as well as in non-producing strains. These factors should be kept in mind when new antibiotics are to be designed with the desired property of overcoming resistance problems usually encountered in bacterial strains that produce infectious diseases.

RESUMEN

La resistencia bacteriana a los antibióticos es un fenómeno complejo que se basa en diferentes tipos de mecanismos dependientes de diversos factores, especialmente relacionados con las cepas resistentes. La inactivación enzimática de los antibióticos parece ser el mecanismo más importante y mejor estudiado. Se han descrito enzimas inactivantes de antibióticos Beta-lactámicos, aminoglicósidos y otros. En la mayoría de los casos, la síntesis de estas enzimas se encuentra codificada por plásmidos que pueden ser transferidos a cepas susceptibles. Sin embargo, los mecanismos que no se basan en la actividad de enzimas específicas también parecen jugar papeles de importancia en el fenómeno de resistencia a los antibióticos, de acuerdo a las evidencias experimentales que se presentan y discuten en el presente trabajo. Entre estos mecanismos, se han demostrado tanto la falla en la unión de los antibióticos a receptores específicos de la célula (especialmente a nivel de la membrana citoplasmática) como la penetración disminuida de las drogas en las células. Estos procesos han sido demostrados tanto en cepas mutantes aisladas en el laboratorio, como en cepas que se han aislado de procesos infecciosos.

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