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REVIEW

Bacterial resistance to penicillins and cephalosporins

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In 1946, 14% of strains of *Staphylococcus aureus* isolated at the Hammersmith Hospital were resistant to benzylpenicillin; by 1947 this figure had risen to 38%, and by 1948 it had become 59% (Barber & Rozwadowska-Dowzenko, 1948). A more recent estimate (Morrison, 1961) puts the proportion of benzylpenicillin-resistant strains of hospital *Staph. aureus* as high as 80%. These figures illustrate the rapid increase in numbers of penicillin-resistant "hospital" staphylococci. It is now thought that the indiscriminate use of penicillins was a major factor responsible for this phenomenon; however, this was not known at the time. Introduction of the penicillinase-stable semisynthetic penicillins, such as methicillin and cloxacillin, to which the most of these benzylpenicillin-resistant strains are sensitive has improved the clinical situation.

The use of broad-spectrum semisynthetic β-lactam drugs, such as ampicillin, carbenicillin and cephaloridine, has made possible the treatment of infections caused by certain Gram-negative organisms which were not previously sensitive to penicillin therapy. At present, most penicillinase-producing strains of *Staph. aureus* are sensitive to the penicillinase-resistant penicillins and most coliform bacteria are sensitive to the broad spectrum β-lactam antibiotics (see Table 1). However, there inevitably exist minority populations which are resistant and, given encouragement, they will become predominant. Therefore, to avoid a repetition of the situation in which benzylpenicillin failed to control the hospital penicillinase-producing staphylococcus, the emergence of resistance must be prevented. A novel method of classification of bacterial resistance is here presented and has been used to summarize the mechanisms by which different bacteria are, or can become, resistant to penicillins and to cephalosporins, to estimate the importance of the various types of resistance, and to indicate how, with some organisms, these may be modified, or prevented. Modes of resistance to penicillins are not yet fully understood, but as many workers are actively studying this problem it is necessary to collect the available data to form a coherent picture of the facts and, more important, of the areas in which further studies would be beneficial. The genetics of penicillinase production will not be discussed in detail, except where strictly applicable to the phenotypic expression of penicillin resistance. These topics have been reviewed by Novick (1965) for staphylococci, Dubnau & Pollock (1965) for bacilli, Eriksson-Grennberg, Boman & others (1965) for escherichia, and Meynell, Meynell & Datta (1968) for R-factors.

Table 1. *Antibacterial "spectra" of several penicillins and cephalosporins*

Organisms	Benzyl-penicillin	Phenoxy-methylpenicillin Phenethicillin Propicillin	Carbenicillin	Ampicillin	Methicillin Nafcillin	Isoxazolyl-penicillins	Cephalothin Cephaloridine Cephaloglycin Cephalexin	Comment
Staph. aureus (non-penicillinase producing)	+	+	+	+	+	+	+	
Staph. aureus (penicillinase producing)					+	+	+	A few strains resistant
Strep. pneumoniae	+	+	+	+	+	+	+	
Strep. faecalis	+	+	+	+	+	+	+	Many strains only marginally sensitive
Strep. pyogenes, Group A	+	+	+	+	+	+	+	
Strep. viridans	+	+	+	+	+	+	+	
N. meningitidis	+	+	+	+	+	+	+	
E. coli			+	+			+	Some resistant strains
K. aerogenes				+			+	Most strains resistant, or marginally sensitive
Pr. mirabilis (penicillinase −)			+	+			+	
Pr. mirabilis (penicillinase +)			+				+	
Pr. rettgeri, morgani, vulgaris			+					
Ps. pyocyanea			+					
Salmonella spp.	+		+	+			+	
H. influenzae	+	+	+	+	+	+	+	

+ Indicates that the drug is likely to be useful because concentrations attainable in the blood during therapy exceed the minimum inhibitory concentrations found *in vitro*.

PHENOTYPIC AND GENOTYPIC RESISTANCE

The sensitivity of a bacterial strain to a penicillin or cephalosporin is governed by two main factors—the resistance of each individual bacterium (the intrinsic resistance), and the capacity of the culture as a whole to produce penicillinase (β-lactamase). The level of intrinsic resistance of a strain normally falls within narrow limits of antibiotic concentration and this is regarded as an indication of "homogeneity" of the culture. Some strains, however, do not conform to this, and there is a wide and non-Gaussian distribution in the intrinsic resistance of individual bacteria of such strains; these are regarded as being "heterogeneous" in their pattern of intrinsic resistance. The significance of these strains will be discussed later. The intrinsic resistance of a strain can be conveniently measured in the laboratory by using a small inoculum (10–100 organisms) by conventional techniques, either on solid or in liquid media. As well as possessing intrinsic resistance, some bacteria may make β-lactamase and these organisms will be even less susceptible to penicillins and cephalosporins than those possessing intrinsic resistance alone. Both intrinsic resistance and penicillinase production are under genetic control; for simplicity, it is convenient to assume that their determinants are simple and independent of each other, and that only two allelic forms exist, namely intrinsically sensitive (S^+)/resistant (S^-), and incapable of destroying penicillin (P^-)/capable (P^+). The ability, or its absence, to destroy penicillin is directly dependent upon the capacity of the bacterial strain in question to produce penicillinase (EC 3.5.2.6.). P^+ strains will destroy penicillin significantly in large, but not in small, inocula. Although bacteria may also produce another penicillin-destroying enzymepenicillinacylase (EC 3.5.1.11.), this enzyme probably plays no significant part in the resistance of bacteria to penicillins for the following reasons. (i) Acylases have low affinities for their substrates, thus, although rapid deacylation can occur *in vitro* at high substrate concentrations, at the much lower penicillin concentrations attainable therapeutically the activity of these enzymes will be drastically reduced. (ii) Acylase activity at physiological pH values is suboptimal. (iii) Production of acylase by bacteria is markedly less at 37° than at lower temperatures. (iv) As the reaction is reversible, deacylation does not go to completion. (v) Both products of the reaction can possess antibacterial properties (see Cole & Sutherland, 1966; Hamilton-Miller, 1966c).

There exist four combinations of alleles which are responsible for sensitivity or significant resistance to penicillins: namely, S^+P^-, S^+P^+, S^-P^- and S^-P^+. Strains

Table 2. *System of classification used*

Class	Genotype: Intrinsic Sensitivity	Genotype: Penicillinase Production	Phenotype
I	S^+	P^-	Sensitive
II	S^+	P^+	Resistant to hydrolysable pencillins Sensitive to non-hydrolysable penicillins
III	S^-	P^-	Resistant
IV	S^-	P^+	Resistant

Notes: Genotype = intrinsic susceptibility to penicillin (small inoculum).
Phenotype = overall susceptibility to penicillin (large inoculum).
Penicillinase production: this merely means production of an enzyme capable of hydrolysing the β-lactam ring in benzylpenicillin. It must not necessarily be assumed that any penicillins other than benzylpenicillin and phenoxymethylpenicillin are hydrolysed by the particular enzyme (see Table 3).

possessing the first of these will clearly be phenotypically sensitive under all conditions and those with the last two will be phenotypically resistant under all conditions. The phenotype of strains belonging to the second class (genotype S^+P^+) will depend on the conditions: when only a few organisms are present (a small inoculum), the strain will be phenotypically sensitive; a large inoculum, however, will contain enough penicillinase to be capable of destroying a significant amount of penicillin, and the strain will thus be phenotypically resistant to hydrolysable penicillins. Resistance is here best defined as the ability of a bacterial strain to multiply in the presence of concentrations of a drug usually attained *in vivo* during its normal therapeutic use; e.g. a strain of *Staph. aureus* not inhibited by 5 μg/ml of methicillin would be a resistant strain, as would be an *Escherichia coli* strain which grows in the presence of 15 μg/ml ampicillin. The scheme by which bacteria can be put into four classes is illustrated in Table 2; this classification will be used as a starting point in the discussion of resistance in this review. The symbol P^+ merely denotes the ability of a strain to produce an enzyme capable of hydrolysing the β-lactam ring in benzylpenicillin.

INTRINSIC RESISTANCE TO β-LACTAM ANTIBIOTICS

Since the precise mode of action of β-lactam antibiotics is not yet fully proven, mechanisms of intrinsic resistance cannot be discussed in precise molecular terms; thus, the subsequent treatment of this topic must inevitably be speculative.

In this discussion it has been assumed that all penicillins and cephalosporins have essentially the same basic mode of action, namely inhibition of mucopeptide biosynthesis. The resulting faultily constructed cell wall is unable to accommodate the cytoplasmic enlargement and internal osmotic pressure of the growing bacterial cell, and partial or complete lysis of the culture, depending upon the exact conditions, follows. Rogers (1967a) calls attention to the role of mucopeptidases in bacterial lysis. In view of the known differences in mucopeptide structure among bacterial species, it seems likely that the detailed mode of action of penicillins and cephalosporins will vary slightly from one species to another, and conversely it is also possible, but less likely, that the detailed modes of action of different β-lactam drugs against a given bacterial strain may vary from one compound to another. Some penicillins appear to have individual side-effects on certain bacteria—viz. nafcillin sensitizes *Staph. aureus* to lysozyme digestion (Warren & Gray, 1967), methicillin increases the permeability of *Staph. aureus* (Rogers, 1967a), and the permeability of coliform bacteria is increased by several penicillins (Smith, 1963a; Hamilton-Miller, 1966a). All these phenomena are caused by concentrations of penicillins which result in less than complete growth inhibition and may be regarded as side-effects due to partial impairment of biosynthesis of cell wall mucopeptide.

The final stage of bacterial mucopeptide biosynthesis is a cross-linking reaction between two peptide chains of peptidoglycan polymer and is thought to be mediated by at least two enzymes, peptidoglycan transpeptidase and D-alanine carboxypeptidase (Izaki, Matsuhashi & Strominger, 1966). The carboxyl group of the D-alanyl-D-alanine moiety of mucopeptide may react with the transpeptidase (see Fig. 1, reaction B1). Subsequent hydrolysis of the peptide bond between the two D-alanine residues would then occur (Reaction B2), leading to release of the terminal D-alanine (Reaction B3) (Strominger, Izaki & others, 1967).

Although the precise site of penicillin action is unknown, two likely hypotheses have been advanced. The first envisages that penicillin acts as a substrate analogue of the

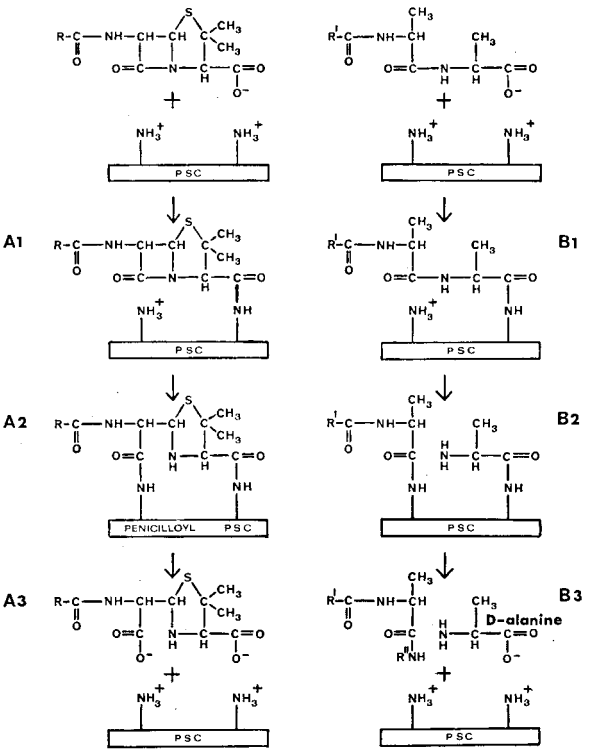

FIG. 1. A_1, A_2 and A_3 show possible reaction sequences of penicillin with the penicillin-sensitive component (PSC). B_1, B_2 and B_3 illustrate the reaction sequences whereby the terminal D-alanyl-D-alanine peptide of peptidoglycan is cross-linked. R–C = O is the acyl side-chain of penicillin. R′–C = O is the remainder of the peptide chain of peptidoglycan. R″NH is the peptide residue of another peptidoglycan chain to which the penultimate D-alanine is cross-linked.

D-alanyl-D-alanine moiety of the peptidoglycan and consequently inhibits the cross-linking reaction (Tipper & Strominger, 1965; Wise & Park 1965). The free carboxyl group of penicillin can mimic the carboxyl group of D-alanyl-D-alanine and become attached to the transpeptidase (see Fig. 1 Reaction A1). This is followed by hydrolysis of the β-lactam bond of penicillin (Reaction A2), which coincides sterically with the peptide bond of D-alanyl-D-alanine, the resulting penicilloylation of the enzyme rendering it inactive for subsequent cross-linking (Tipper & Strominger, 1965). Of the two enzymes concerned in cross-linking, the transpeptidase is the more likely candidate since penicillin inactivates it at growth inhibitory concentrations; D-alanine carboxypeptidase is also inhibited by penicillin but only reversibly and at levels inconsistent with growth inhibitory concentrations (Strominger & others, 1967).

The second hypothesis proposes that penicillin interacts with a peptidoglycan precursor binding site on the bacterial cell membrane thus preventing the enzymes necessary for completion of peptidoglycan synthesis from reaching the substrate (Rogers, 1967b). These two views may well turn out to be one and the same thing when the precise nature of the cross-linking reaction is more fully understood.

For the purposes of this discussion it is irrelevant which of these theories is correct; the component (be it enzyme or membrane) with which penicillin combines to exert its antibacterial influence will be called merely the "penicillin-sensitive component" (PSC).

The reaction between PSC and penicillin (P) may be represented thus:

$$PSC + P \rightarrow PSC\text{–}P$$

the velocity of this reaction $= k_1[P][PSC]$. The penicillins are assumed to bring about this reaction by penicilloylation (Fig 1, reaction A2), and hence the reaction is, on chemical grounds, irreversible.

For balanced growth to occur it is postulated that a certain concentration ($= [PSC]_{min}$) of free PSC must be present in the cell. The action of penicillin is to reduce the total concentration of PSC ($= [PSC]_{total}$) by sequestering PSC in the form of the inactive complex PSC–P. It can be seen that, under a given set of conditions, $[PSC]_{free} = [PSC]_{total} - [PSC\text{–}P]$; if $[PSC]_{free} > [PSC]_{min}$, growth can occur, while if $[PSC]_{free} < [PSC]_{min}$, no growth will occur. For the special case $[PSC]_{free} = [PSC]_{min}$, penicillin is present at the minimum inhibitory concentration ($= [P]_{mic}$). It follows that any process whereby $[PSC]_{free}$ can be increased will also increase $[P]_{mic}$, and thus by definition the intrinsic resistance will be increased.

It also follows that decreases in either k_1 or [P] (see (2) below), for example by mutation, would reduce the rate of reaction, and thus these bacteria would possess a higher level of intrinsic resistance. Factors which could contribute towards decreases in these two parameters will be discussed below.

(1) Decrease in k_1; k_1 is a measure of the "affinity" of a penicillin for its active site, and as such has been discussed by Rolinson (1965) *vis-à-vis* whole cells of *Staph. aureus* and *E. coli*, and by Izaki & others (1966) for a cell-free system of PSC from *E. coli*. It can be seen that a slight change in the chemical nature of PSC might radically alter its affinity for a penicillin while not affecting its ability to participate in the cross-linking reaction; such a "qualitative" change in PSC could be responsible for the intrinsic resistance of the bacterium which contained it.

(2) Decrease in [P]. (*i*) Change in permeability; if the ease with which P could approach PSC were reduced, the overall effect, all other things being equal, would be to decrease [P], and thus to bring about an increase in the intrinsic resistance of the strain involved. There is evidence for the existence of a substantial permeability barrier to benzylpenicillin in the intrinsically sensitive Oxford strain (Rogers, 1967b), and to methicillin in the intrinsically sensitive 524 SC strain of *Staph. aureus* (Rogers, 1967a). (*ii*) Ability of PSC-P to dissociate. Dissociation of PSC-P will clearly ease the metabolic block imposed by an inhibitory concentration of P, by releasing free sites to continue cell wall synthesis. As stated above, such a dissociation cannot be a mere reversal of reaction A2 (Fig. 1), as the cyclization of a penicilloate to a penicillin requires highly unusual chemical conditions (Sheehan & Henery-Logan, 1962); the only possible route for PSC–P to dissociate seems to be that represented by reaction A3 which is the chemical notation for

$$PSC\text{–}P \rightarrow P' + PSC,$$

where P′ represents penicilloate; the rate of dissociation is $k_2[PSC\text{–}P]$. It can thus be seen that the overall velocity of sequestration of PSC has been reduced, under these circumstances, to $k_1[P][PSC] - k_2[PSC\text{–}P]$, with a corresponding decrease in bacterial sensitivity, and thus, PSC is acting as a penicillinase (see Pollock, 1967).

There is also the following possibility:

(3) Increase in $[PSC]_{total}$; assuming that both the supply of penicillin and the amount of PSC are rate-limiting [as has been indicated by the experiments of Rogers (1967b), who showed that the reaction between P and PSC obeyed second-order

kinetics in *Staph. aureus* Oxford], any increase in the absolute amount of PSC will overcome the metabolic block imposed by penicillin. Thus, an increased synthesis of PSC will result in an increase in the intrinsic penicillin resistance on the part of a particular bacterium; such increased synthesis could be brought about by, for instance, an increased sensitivity of the relevant feedback mechanism. This type of "quantitative" alteration in PSC production could clearly take place either gradually, by adaptation, or stepwise, by mutation followed by selection of the resistant mutants.

Unfortunately, there is little or no evidence about which of these means of acquiring resistance is being used in any particular case. There is no doubt that the penicillinase mechanism (see above) is responsible for resistance to benzylpenicillin in *Staph. aureus*. There is some circumstantial evidence (Hugo & Stretton, 1966; Redai, Rethy & Vaczi, 1967; Bruns, 1967) suggesting that changes in the intrinsic resistance of *Staph. aureus* strains may be caused by changes in the lipid content of the bacteria; such changes can readily occur without any deleterious effect on growth patterns or viability (Vaczi, Redai & Rethy, 1967). It is clear that cultures composed of individuals with differing fatty acid compositions, and hence differing permeabilities to penicillins, offer a potentially rich field on which natural selection may work. It is interesting that acquisition of resistance to penicillins can bring about some radical changes in surface structures (e.g. change in phage type of *Salmonella panama*—Guinee, Scholtens & Willems, 1967), which may reflect alterations in permeability barriers.

MANIFESTATIONS OF PENICILLIN-RESISTANCE BY DIFFERENT BACTERIAL SPECIES

We have found it convenient to consider Gram-positive and Gram-negative species separately, for their resistance and sensitivity to penicillins.

(a) GRAM-POSITIVE BACTERIA

Class I. *Non-penicillinase-producing, intrinsically sensitive strains* (S^+P^-)

Group A streptococci (*Streptococcus pyogenes*) are typical members of this class. A survey in Czechoslovakia (Jelinkova & Jelinek, 1965) showed that the mean sensitivity of strains isolated between 1952 and 1964 has remained at about 0·015 units/ml benzylpenicillin: there was no indication of any increase in penicillin resistance over this period. Other examples of species falling into this class are non-penicillinase producing strains of *Staph. aureus* (typified by the Oxford strain), and *Listeria monocytogenes*.

Infections caused by the bacteria in this class usually respond to treatment with benzylpenicillin. Failure to respond to such treatment can usually be attributed to either accessibility difficulties (e.g. penetration of penicillin into vegetations on heart valves) or to concomitant infections by penicillinase-producing organisms (Tacking, 1954, but see also Lagerholm, Lodin & Nystrom, 1966, Quie, Pierce & Wannamaker, 1966, Markowitz, Kramer & others, 1967).

Class II. *Penicillinase-producing, intrinsically sensitive strains* (S^+P^+)

The typical hospital staphylococcus (Barber, 1947) is found in this class; such organisms are characteristically of phage group III, resistant to mercuric salts and to tetracycline (Richmond, Parker & others, 1964). Penicillin resistance is due to penicillinase production (Barber, 1947), and the great increase in the proportion of these strains shortly after the large-scale introduction of penicillin therapy (see Introduction) reflects the selection of penicillinase-producing strains because of their ecological

advantage over non-penicillinase-producing strains in the presence of a hydrolysable penicillin such as benzylpenicillin. There has been no case reported in which a penicillinase-producing strain of *Staph. aureus* has arisen *de novo* from a penicillinase-negative parent, i.e. a Class I to II mutation (see Fig. 2).

Penicillinase-producing *Staph. aureus* strains may have arisen from penicillinase-positive coagulase-negative strains (such as *Staph. albus*), which have gained coagulase and other factors concerned with virulence. This could occur *in vivo* either by mutation, transduction (Novick & Morse, 1967) or by a mechanism similar to exhaltation of virulence.

Organisms in this class show a typical inoculum-size effect, i.e. in small inocula they are many times more sensitive than in large inocula (Luria, 1946). That it is the intrinsic resistance of such strains that is measured by determining the minimum inhibitory concentrations for small inocula, is demonstrated by the fact that the minimum inhibitory concentration for a small inoculum of *Staph. aureus* E3T (a penicillinase-negative variant of E3—Knox, 1960; Knox & Smith, 1961) is the same as that for a small inoculum of the penicillinase-producing parent strain, E3. Moreover, Class II strains of *Staph. aureus* will, by virtue of this intrinsic sensitivity, be susceptible to β-lactam antibiotics which are not hydrolysed by staphylococcal penicillinase, such as methicillin, the isoxazolyl penicillins, quinacillin and certain cephalosporin derivatives.

Spontaneous loss of P^+ occurs *in vitro* at a rate of between 10^{-4} (Novick, 1963) and 10^{-3} (Seligman, 1966) and is therefore a Class II to I mutation (see Fig. 2). The rate of loss of P^+ is increased under "adverse conditions" such as the presence of methicillin (as in the conversion of E3 to E3T—Knox, 1960; Knox & Smith, 1961), or growth at 44° (May, Houghton & Perret, 1964). Gorrill & McNeil (1965) have shown that this change can also occur *in vivo*.

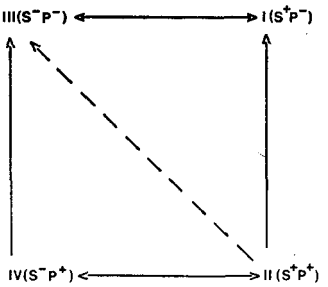

FIG. 2. Summary of changes that can occur in the intrinsic sensitivity and the capacity to produce penicillinase in *Staph. aureus* strains. P^+ = capable of producing penicillinase. P^- = not capable of producing penicillinase. S^+ = intrinsically sensitive. S^- = intrinsically resistant.

Kjellander, Klein & Finland (1963) have investigated several benzylpenicillin-resistant strains of *Staph. albus*, and found that most were resistant by a penicillinase-type mechanism; this bacterial species has been found to cause an alarmingly high mortality in cases in which it has been isolated from blood cultures (Finland, Jones & Barnes, 1959).

Recent investigations have shown that *Mycobacterium tuberculosis* produces penicillinase, and that this factor plays a not unimportant part in its resistance to penicillins (Dufour, Knight & Harris, 1966; Kasik & others, 1967). Thus, *Myco. tuberculosis* (human, bovine and avian strains) belongs in Class II; Kasik, Weber &

others (1966) have demonstrated that tuberculosis in mice may successfully be treated with a synergistic mixture of dicloxacillin and benzylpenicillin.

Class III. *Penicillinase-negative, intrinsically resistant strains* (S^-P^-)

Strains belonging to the Runyon Group III of "anonymous" mycobacteria, the so-called Battey strains, are resistant to penicillins and do not produce penicillinase (Dufour & others, 1966); a reason for this intrinsic resistance may be the inability of the drugs to penetrate the lipid-rich cell wall.

Strains of Group A *Strep. pyogenes* resistant to benzylpenicillin have been obtained by laboratory training techniques (Yoshioka & Kunii, 1965); such strains do not produce penicillinase, and are thus Class III strains. The resistant mutants had lost some of their virulence, and resemble some artificially produced Class III *Staph. aureus* (see below).

Group D-streptococci (enterococci) have a natural intrinsic resistance to most penicillins, a fact which often complicates the treatment of subacute bacterial endocarditis caused by this species. However, it seems that streptomycin acts synergistically with benzylpenicillin or ampicillin against certain strains of enterococci (Hewitt, Seligman, & Deigh, 1966; Simon, 1967).

Stirland & Shotts (1967) have reported that strains of *Strep. viridans* having an enhanced resistance to benzylpenicillin (Class III strains) may be selected during therapy; if such a phenomenon continues, the result will clearly be the large-scale emergence of penicillin-resistant *Strep. viridans* strains.

Pneumococci resistant to benzylpenicillin have been obtained by *in vitro* training (McKee & Houck, 1943; Eriksen, 1946; Gunnison, Fraher & others, 1968). In some cases, but not all, such strains—which had increased their resistance by from five to eighty fold—had lost mouse virulence. Hansman & Bullen (1967) isolated from a patient a type 23 pneumococcus which was relatively resistant to benzylpenicillin (MIC 0·6 μg/ml, compared with 0·03 μg/ml for typical strains) and ampicillin; this appears to be the only case of a wild pneumococcal strain showing some degree of penicillin-resistance.

Class III strains of *Staph. aureus* fall into three categories; the first consists of an interesting type of organism—penicillinase-producing strains of *Staph. aureus* (Class II) that have been trained in the laboratory to "tolerance" to a penicillin, by means of serial sub-culture in increasing concentrations of the drug (Barber, 1961; Knox & Smith, 1961). Such organisms are resistant to the penicillin upon which they were grown (and also display cross-resistance to other penicillins—Knox, 1960—and to cephalothin—Godzeski, Brier & Pavey, 1963), solely by an intrinsic mechanism, as they have lost their capacity to produce penicillinase. It seems that this Class II to III mutation (see dotted line Fig. 2) is a summation of two separate mutational events e.g. II to I and I to III. These changes also involve loss of virulence (Barber, 1961; Knox & Smith, 1961). It thus appears, on balance, unlikely that strains of *Staph. aureus* belonging to this group can be of great clinical significance. The increased intrinsic resistance as a consequence of laboratory training techniques may be caused by a decrease in permeability of the membrane to the drug, or it may involve the use of an alternative metabolic pathway to bypass the step in cell wall synthesis which is inhibited by the penicillin. In the context it is interesting that a strain of *Pediococcus cerevisiae* has been obtained (White, 1968) which is dependent upon methicillin for growth.

The second category of Class III strains of *Staph. aureus* is comprised mainly of strains that occur naturally; Farkas-Himsley, Soeprihatin & Goldner (1961) reported having isolated from human and animal sources seven strains of *Staph. aureus* resistant to both methicillin and cloxacillin, and that did not produce penicillinase. Seligman (1966) reported that the natural rate of loss of the ability to produce penicillinase among methicillin-resistant strains of *Staph. aureus* was of the order of 10^{-3} (i.e. one organism per 1000 is no longer able to produce the enzyme) (i.e. a class IV to III mutation see Fig. 2), and thus it appears possible that the strains isolated by Farkas-Himsley and his colleagues represent such loss mutants, the mutation possibly having occurred *in vivo*.

The third category of Class III strains of *Staph. aureus* contains S^+P^- strains, for example mutants of E3T or the Oxford staphylococcus which have been obtained by selecting for intrinsic resistance in the laboratory, i.e. a Class I to III mutation, see Fig. 2. Barber (1961) reports that such strains are of decreased virulence and are often unstable in the absence of penicillin, reverting from Class III to Class I strains spontaneously (Fig. 2). Therefore it is doubtful if these Class III strains are of clinical significance.

Class IV. *Penicillinase-producing intrinsically resistant strains* (S^-P^+)

Barber (1964) investigated 59 strains of naturally-occurring methicillin-resistant *Staph. aureus*, isolated in Britain, France and Denmark; all belonged to closely related phage types (Group III), and all produced penicillinase. It has been established that the resistance of large inocula of such strains is because they are "heterogeneous", i.e. they contain a small proportion of individuals with a high intrinsic (genotypic) resistance (Knox & Smith, 1961; Sutherland & Rolinson, 1964; Chabbert, Baudens, & others, 1965; Seligman & Hewitt, 1965). Methicillin-resistant staphylococci are also resistant to isoxazolyl penicillins (Eriksen & Erichsen, 1964) and to quinacillin (Smith, Hamilton-Miller & Knox, 1964). It has been claimed (Stewart & Holt, 1963; Eriksen & Erichsen, 1964) that the resistance of these strains is due to an increased ability on their part to destroy methicillin and isoxazolyl penicillins; however, Knox & Smith (1963) and Ayliffe & Barber (1963), using the same strains, showed that methicillin resistance was not caused by increased 'methicillinase' activity, but was due to the intrinsic mechanism. Seligman (1966) showed that penicillinase-negative mutants of 5 methicillin-resistant, penicillinase-producing *Staph. aureus* strains retained practically *in toto* their parents' resistance to methicillin. This Class IV to III mutation is shown in Fig. 2. This author, Dyke, Jevons & Parker (1966), and Hewitt & Parker (1968), suggest that a high grade resistance to methicillin (up to 500 μg/ml) is entirely due to an intrinsic mechanism.

The origin of Class IV strains of *Staph. aureus* is not known. However, laboratory training procedures can enable Class II strains to become intrinsically resistant to penicillins while retaining their P^+ characteristic as well as full virulence (Barber, 1961); an example of a Class II to IV mutation (see Fig. 2) is the change of strain 13137 to 13137/1000 (Knox & Smith, 1961). Whether this change can occur *in vivo* (e.g. during the course of treatment of a patient with a penicillin) is unknown . There is no *prima facie* reason why it should not, though it has not occurred (Stewart, 1960), even under favourable conditions (Elek & Fleming, 1960). However, since it is known that Class IV strains isolated *in vitro* are unstable in the absence of penicillins, losing their

intrinsic resistance whilst retaining their P^+ characteristic, similar conditions possibly apply *in vivo* and this would prevent their large scale emergence.

Methicillin-resistant strains generally show cross-resistance to semi-synthetic cephalosporins (Barber & Waterworth, 1964a; Hamilton-Miller & Ramsay, 1967; Hewitt & Parker, 1968). Although it has been shown (Hamilton-Miller & Ramsay, 1967) that cephaloridine is less stable to staphylococcal penicillinase than cephalothin, methicillin-resistant P^+ strains are nonetheless more sensitive to cephaloridine than to cephalothin; this is accounted for by the increased intrinsic activity of the former drug [due to its possession of a pyridinium moiety (see Hale, Newton & Abraham, 1961)], which outweighs its greater lability to penicillinase.

Methicillin-resistant strains of *Staph. aureus* maybe on the increase both in the United Kingdom and in France. Jevons (1961) stated that 3 out of 5,440 strains isolated between October and December 1960 (i.e. before the use of methicillin) were methicillin-resistant, an incidence of 0·055%; a further survey by Jevons, Coe & Parker (1963), analysed 27,479 strains isolated between October 1960 and September 1962; 102 (0·48%) were resistant to methicillin. Later, Colley, McNicol & Bracken (1965), who investigated strains isolated between September 1962 and April 1964, found an overall incidence of methicillin resistance of 4·1% and Turner & Cox (1967) have observed incidences of infection with methicillin-resistant strains of 5·4% in 1963, rising to 16·6% in 1965; however, this latter set of figures is not strictly comparable to those given earlier, as the incidence was reported in terms of total multiple-resistant strains. Chabbert, Baudens & others (1965) found that as many as 19% of strains of *Staph. aureus* isolated in Paris hospitals during 1963 were potentially resistant to methicillin and oxacillin, as they contained a small but significant proportion (0·1–10%) of individuals with a markedly increased intrinsic resistance. A more recent survey in Paris (Bastin, Worms & Acar, 1967) shows that 36% of cases of staphylococcal septicaemia occurring between January 1963 and October 1966 were caused by methicillin-resistant strains. Breitfellner (1967), however, reports no increase, between 1960 and 1966, in the incidence of oxacillin-resistant strains isolated in Vienna. Kjellander & others (1963) reported a relatively high incidence of resistance to methicillin and to oxacillin among penicillinase-producing *Staph. albus* strains; such resistance was shown to be intrinsic, as neither penicillin was destroyed by *Staph. albus* penicillinase.

Over 300 methicillin-resistant *Staph. aureus* strains have been isolated in Great Britain (Colley & others, 1965), and further strains have been found in France (Chabbert & others, 1965), Germany (Stille & Brandis, 1965), Denmark (Eriksen & Erichsen, 1964), Sweden, Spain, Argentina and U.S.A. (Isenberg, 1965; Benner, Bennett, & others, 1965). It is interesting that relatively few strains have been isolated in U.S.A., where large scale use of methicillin and the isoxazolyl penicillins is comparable to that in Europe. Many methicillin-resistant strains belong to phage Group III; it was originally thought that methicillin resistance only occurred in this phage group, but Isenberg (1965), Benner & Morthland (1967) and Chabbert & Pillet (1967) showed that this was not so.

There have been very few serious infections in Great Britain caused by methicillin-resistant *Staph. aureus* strains—one death was reported by Stewart & Holt (1963), five by Colley & others (1965), and two serious infections by Harding (1963).

The fact that methicillin-resistance is due to the heterogeneity of bacterial cultures tends to lessen its clinical implications: a patient with an infection caused by such a

heterogeneous strain may still be treated with methicillin with a reasonable chance of success (see Chabbert & others, 1965; Bastin & others, 1967; Turner & Cox, 1967). Most of the bacteria will succumb to therapeutically attained concentrations of the drug, and the small numbers of resistant cells remaining may be adequately dealt with by the body. However, this view is not held by Benner & Kayser (1968), who recommend vancomycin treatment.

Several workers (e.g. Barber, 1964; Sutherland & Rolinson, 1964; Seligman, 1966; Hamilton-Miller & Ramsay, 1967) have reported that methicillin-resistant strains of *Staph. aureus* contain a significant proportion of colonial variants, particularly when the bacteria are grown in the presence of methicillin. These colonies resemble G-forms, and it seemed possible at one stage that they represent a type of L-form lacking both cell wall and sensitivity to penicillins. If this were so, it would be a potentially dangerous situation, as such degenerative forms could act as a reservoir of staphylococci that could cause recrudescence of infection on the cessation of methicillin treatment. However, examination of "G-like" colonies by Bulger & Bulger (1967) showed them to be composed of staphylococci with normal cell walls; and Barber (1964) had shown that the incidence of such colonial variants was greatly reduced by the incorporation of a high salt concentration into the growth medium. This suggested that these colonies suffer from some osmotic imbalance. Knox & Smith (1961) found that a laboratory-trained methicillin-resistant strain E3/500 (made from a Class II strain, E3) had lost its penicillinase, changed to the G-form and gained intrinsic resistance simultaneously. It had also lost a significant amount of its mouse virulence.

It seems probable that many infections caused by methicillin-resistant staphyloccoci have been resolved without the true nature of the infecting organism becoming known; the laboratory diagnosis of methicillin-resistance cannot normally be made satisfactorily by the disc method (Kayser, 1967) and the more laborious tube dilution test requires 48 h incubation before the result can be read (Sutherland & Rolinson, 1964). Benner & Morthland (1967) recommend vancomycin for the treatment of infections with known methicillin-resistant strains of staphylococci, and Bulger (1967) favours combinations of kanamycin with either methicillin or cephalothin. Kanamycin has also been used by French clinicians (Bastin & others, 1967).

(b) GRAM-NEGATIVE BACTERIA

The causes of penicillin resistance in Gram-negative species are not as clear as in Gram-positive species partly because intensive investigations of Gram-negative organisms did not start until the therapeutic introduction of ampicillin in 1961. At first most workers assumed that ampicillin was hydrolysed as readily by penicillinases from Gram-negative bacteria as by staphylococcal penicillinase, and hence that all penicillinase-producing Gram-negative bacteria were considered ampicillin-resistant because they destroyed the drug (Class II strains). However, it is now evident that the penicillinases from Gram-negative organisms often have properties very different from those of Gram-positive species, and each Gram-negative species may produce a unique penicillinase (Smith & Hamilton-Miller, 1963). Some of the properties of the penicillinases from various species of bacteria are given in Table 3. It can be seen, for instance, that some penicillinase-producing *E. coli* strains do not hydrolyse ampicillin (Smith, 1963a); nevertheless, it has often been assumed that such *E. coli* strains will be resistant to ampicillin by the drug inactivation mechanism.

Table 3. *Some properties of penicillinases from different bacterial species*

	Gram-positive bacteria				Gram-negative bacteria		
					"Penicillinase-type"		"Cephalosporinase-type"
SUBSTRATE SPECIFICITY	*Staph. aureus*	*B. cereus* β-*lactamase* I	*B. cereus* β-*lactamase* II	*B. licheniformis*	*Klebsiella, Proteus mirabilis*	*Aerobacter cloacae*	*E. coli, Enterobacter spp., Pr. vulgaris, Pr. rettgeri, Pr. morgani, Ps. pyocyanea*
Hydrolysis of:							
Benzylpenicillin, phenoxymethylpenicillin.	Rapid	Rapid	Rapid	Rapid	Rapid	Rapid	Rapid
Phenethicillin, phenbenicillin, propicillin.	Rapid	Rapid	—	Rapid	Rapid	Rapid	Very slow
6-Aminopenicillanic acid.	Slow	Slow	—	Slow	Rapid	Rapid	Very slow
Ampicillin.	Very rapid	Rapid	Rapid	Rapid	Very rapid	Very rapid	Very slow
Methicillin, isoxazolyl penicillins, quinacillin.	Very slow	Very slow	Rapid	Slow	Very slow	Rapid	Very slow
Cephalosporin C, cephaloram, cephaloridine, cephalothin.	Very slow	Very slow	Rapid	Slow	Slow	Slow	Very rapid
INDUCIBILITY	Induced at low inducer concentrations	Induced at low inducer concentrations	Inducible	Induced at low inducer concentrations	Not inducible	Not inducible	*E. coli* not inducible Others induced by high inducer concentrations
AFFINITY FOR COMPETITIVE INHIBITORS	Very high for semi-synthetic cephalosporins; very low for methicillin	Low for methicillin and isoxazolyl penicillins		Low for methicillin and isoxazolyl penicillins	Very high for methicillin slightly lower for cloxacillin		Very high for cloxacillin slightly lower for methicillin
MOLECULAR WEIGHT	29,600	30,700	—	23,000	Possibly similar to that of R-Factor TEM (= 16,700)		

Data are taken from Citri & Pollock (1966), and from Smith (1963a, b, and unpublished experiments), Smith & Hamilton-Miller (1963), Hamilton-Miller & Smith (1964), Hamilton-Miller (1966b, 1967 and unpublished experiments), Ayliffe (1965), Hennessey (1967), Kuwabara & Abraham (1967).

An attempt has been made to assign Gram-negative strains to four classes, as indicated on Table 2, as with Gram-positive strains.

Class I. *Penicillinase-negative intrinsically sensitive strains* (S^+P^-)

Moraxella, Salmonella, Shigella, H. influenzae, N. gonorrhoeae and *N. meningitidis*, together with non-penicillinase-producing strains of *E. coli* and *Proteus mirabilis*, are normally all sensitive to ampicillin, and infections caused by them are usually cured by ampicillin therapy. The Neisseria behave more like Gram-positive cocci being more sensitive to benzylpenicillin than ampicillin. Failure in the treatment of infections caused by Class I Gram-negative bacteria can often be ascribed to penicillin-destroying strains (e.g. P^+ strains of *Staph. aureus* or *Klebsiella aerogenes*) present at the same time —see Kjellander & Finland (1963).

Class II. *Penicillinase-producing intrinsically sensitive strains* (S^+P^+)

If a bacterial strain possesses a penicillinase capable of hydrolysing ampicillin this does not necessarily mean that resistance is due to the possession of such an enzyme (see Ayliffe, 1963). Hamilton-Miller (1965) found that penicillinase activity accounted for the observed clinical resistance of only two out of 12 penicillinase-producing *K. aerogenes* strains; the remainder were also intrinsically resistant (Class IV strains). Sutherland (1964) found, similarly, that only in some cases is penicillinase production solely responsible for resistance of penicillinase-producing Gram-negative bacteria to ampicillin. Barber & Waterworth (1964b) have suggested that P^+ strains of *Pr. mirabilis* are resistant to ampicillin by a penicillinase-type mechanism.

Increased penicillinase production in mutants selected for increased overall resistance has been assumed without further tests to be responsible for the overall resistance. But it is essential that the intrinsic resistance (MIC with small inoculum) of the parent and resistant strains is measured before the resistance increase is so attributed. A rise in intrinsic resistance sometimes takes place at the same time as an increase in penicillinase activity (e.g. Kabins, Sweeney & Cohen, 1966) and it is often mistakenly assumed that an inoculum size effect is proof of a penicillinase-type resistance—this is true only if the bacterial population under investigation is homogeneous.

Where ampicillin-resistance is due to penicillinase-mediated destruction of the drug, it may be possible, by inhibition of the enzyme using a competitive inhibitor such as methicillin or cloxacillin, to render such strains phenotypically sensitive to ampicillin (Hamilton-Miller, Smith & Knox, 1964; Sutherland & Batchelor, 1964). A similar situation exists in Class II strains phenotypically resistant to the cephalosporin antibiotics: Sabath & Abraham (1964) found that cloxacillin inhibited the hydrolysis of cephalosporins by a strain of *Pseudomonas pyocyanea*, and also that the presence of this penicillin greatly increased the sensitivity of the strain to cephalosporin C, cephalothin and cephaloridine. Results with *K. aerogenes* and *E. coli* (Hamilton-Miller, Smith & Knox, 1965) indicate that their penicillinases when hydrolysing cephaloridine are more susceptible to inhibition by methicillin, cloxacillin or quinacillin than when hydrolysing benzylpenicillin. It is sometimes possible to overcome resistance in Class II strains by combined therapy of methicilin or cloxacillin with ampicillin or benzylpenicillin (see e.g. Sabath & others, 1967). It is essential for the success of such combined therapy that detailed laboratory studies should precede and accompany treatment. These should show that the bacterial strain in question is inhibited *in vitro* by the appropriate β-lactam drugs in concentrations which are actually attained in the patient being treated. Uncontrolled trials of such combined therapy may lead to a potentially

valuable clinical tool falling into disrepute, as clinical failures will undoubtedly occur if treatment is given in an arbitrary fashion.

Class III. *Penicillinase-negative, intrinsically restraint strains* (S^-P^-)

The list of Gram-negative bacteria falling into Class III has declined steadily owing to the consecutive introduction of ampicillin, semi-synthetic cephalosporins (cephalothin, cephaloridine, cephaloglycin and cephalexin) and carbenicillin into medicine. As knowledge accumulates, it seems more and more likely that those Gram-negative bacteria resistant to the newer semisynthetic β-lactam drugs produce penicillinase (again it must be stressed that this is not necessarily a cause-and-effect relationship); if this view is substantiated, very few Class III Gram-negative bacteria exist. At present, *Serratia marcescens* (Hugo & Furr, 1967) and *Acinetobacter anitratus* (Thornley, 1967), appear to be genuine members of this Class. However, for practical purposes, such strains as *Ps. pyocyanea* and the indole-producing *Proteus* species may most conveniently be included in Class III because under physiological conditions they do not normally produce penicillinase. This enzyme is only formed after induction, involving growth in the presence of high penicillin concentrations (of about 1 mg/ml, Hamilton-Miller, 1963b; Ayliffe, 1964; Jago, Migliacci & Abraham, 1963), and so these strains would be expected to produce significant amounts of penicillinase *in vivo* only when present in the urinary tract of patients treated with β-lactam drugs. Furthermore, it is not clear whether all strains of these species are capable of producing penicillinase.

Sutherland, Slocombe & Rolinson (1964) found that a Class I strain of *Salm. paratyphi* B could be trained to ampicillin resistance (presumably this was a Class III resistance, although the authors did not state whether penicillinase was produced) and resistant mutants so obtained showed strong cross-resistance to chlortetracycline and chloramphenicol: this suggests a generalized decrease in permeability, limiting the access of these drugs into the bacteria. Jones (1966) made similar findings using *in vitro* trained P^- *E. coli* strains.

Certain bacterial species, notably *N. gonorrhoeae*, appear to have gradually built up intrinsic resistance over the years, and now seem about to become clinically resistant. Hejzlar & Vymola (1965) and Amies (1967) have indicated the alarming rise in the *in vitro* resistance of gonococci to benzylpenicillin recently, an increase paralleled by a rise in the failure rate for the single-dose treatment of gonorrhoea (Kjellander & Finland, 1963; Oller, 1967). As gonococci have not yet been shown to produce penicillinase, this increased resistance is another example of an *in vivo* selection of Class III mutants from a predominantly Class I population.

Class IV. *Penicillinase-producing intrinsically-resistant strains.* (S^-P^+)

The penicillinase-producing *Ps. pyocyanea*, indole-producing *Proteus* and *Enterobacter* spp. can be regarded as Class IV strains under certain circumstances (e.g. when they are infecting the genito-urinary tract, because ambient concentrations of penicillins or cephalosporins are then likely to be high enough to cause induction of the enzyme). The recent introduction of carbenicillin (Acred & others, 1967) has made possible the use of a penicillin to treat systemic infections by *Pr. vulgaris*, *Pr. morgani* or *Pr. rettgeri*; this penicillin is much more intrinsically active against these bacteria than any other β-lactam drug, and is claimed to be unaffected by their penicillinases.

Smith (1963a) found that, by training an intrinsically ampicillin-sensitive penicillinase-producing (Class II) strain of *E. coli* to ampicillin resistance *in vitro*, he obtained a

Class IV strain. He also found that in the process of converting intrinsic sensitivity to resistance the penicillinase-producing capacity of the strain was greatly increased, even though the penicillinase did not hydrolyse ampicillin. Similar findings have been made for *E. coli* by Percival, Brumfitt & deLouvois (1963) and by Seligman & Hewitt (1965). In all cases, conversion of genotype S^+ to S^- was accompanied by increased penicillinase production. Jones (1966), however, was able to obtain P^-, intrinsically resistant strains (Class III) of *E. coli* by laboratory training, and Barber & Waterworth (1964a) reported similar results using both *E. coli* and a penicillinase-producing strain of *Pr. mirabilis*. The latter authors observed increased production of penicillinase by a laboratory trained *K. aerogenes* strain. A genetic analysis of ampicillin resistance in mutants of *E. coli* K12 has been made by Eriksson-Grennberg & others (1965).

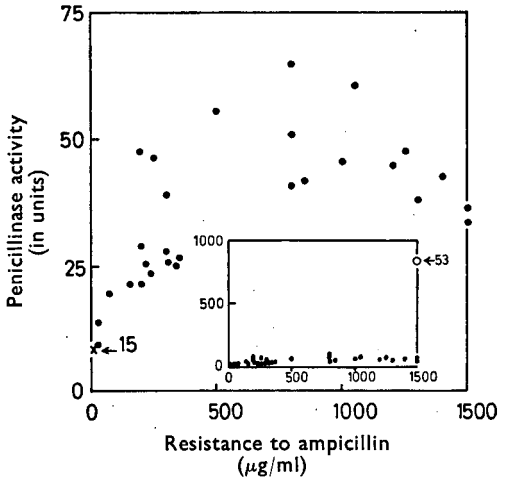

FIG. 3. The penicillinase activities of various mutants derived *in vitro* from *A. cloacae* 15 (× on graph) are plotted against the ampicillin concentrations from which they were isolated. Also shown is the penicillinase activity of *A. cloacae* 53 (○ on graph) which was isolated *in vivo*. Penicillinase activities are expressed as nmol of benzylpenicillin destroyed/min at pH 6·0 and and 37° C in presence of M/40 sodium phosphate buffer by a disrupted suspension of bacteria which previously contained 10^9 viable units.

The role of penicillinase production in acquired penicillin resistance is clarified by the following observations (J. T. Smith, 1964, unpublished observations): a strain of *Aerobacter cloacae* (reference number 15), originally isolated from a patient undergoing treatment with ampicillin, was sensitive to ampicillin (MIC = 10 μg/ml), and produced a small amount of penicillinase; a further strain (reference number 53), later isolated from the same patient, was highly resistant to ampicillin (MIC = 15 mg/ml) and produced almost 100 times as much penicillinase as strain 15. The two strains were serologically and biochemically identical, and the penicillinases produced by both also appeared identical; the action of this enzyme was inhibited by sulphydryl-blocking reagents, and it had an unusually wide substrate specificity profile (Smith, 1963b; Smith & Hamilton-Miller, 1963). It is thought that strain 53 is a mutant of strain 15 which acquired ampicillin-resistance *in vivo*. To determine whether it is the higher penicillinase content of strain 53 which is solely responsible for its greater resistance to ampicillin (for this penicillinase does hydrolyse ampicillin), a program of training the parent strain (15) by serial subculture on agar containing increasing concentrations of ampicillin was undertaken. At various stages in this process, colonies were removed from the plates which contained, at that time, the highest level of ampicillin permitting

growth, and were tested to determine their level of penicillinase. The results are shown in Fig. 3. At first it seemed that the various mutants of strain 15 had gained resistance in proportion to their increase in penicillinase activity, at least in the series of mutants for which the MIC of ampicillin were up to 1 mg/ml. However, from the inset graph, it can be seen that the amount of penicillinase gained by these mutants is insignificant compared to the enormous increase in activity from the original sensitive strain 15, to the mutant strain 53 obtained *in vivo* (these are marked with X and O on the main and inset graphs, respectively). It is probable that the mutants obtained *in vitro* are resistant due to an increase in their intrinsic resistance (and therefore belong to Class IV of the present classification), and that an incidental rise in penicillinase activity has also occurred, as has been previously discussed for *E. coli.* Further substantiation of the intrinsic type of resistance is indicated by those mutants resistant to concentrations of ampicillin greater than 1 mg/ml—these mutants tend to have less penicillinase than the mutants which are less resistant to ampicillin. It is also clear that the acquisition of ampicillin resistance by strain 53 cannot have come about by a process analogous to the *in vitro* training. Seligman & Hewitt (1965) have also reported a step-wise increase in intrinsic resistance as a result of training an *E. coli* strain to ampicillin resistance; there was a concomitant rise in penicillinase production.

A further example of Class IV resistance is given by Benner, Micklewait & others (1965) who observed that in certain strains of Aerobacter, resistance to cephalothin and to cephaloridine was not necessarily based on production of "cephalosporinase".

A characteristic feature of Gram-negative penicillinase-producing bacteria is their permeability barrier, which limits the access of certain penicillins (notably benzylpenicillin and ampicillin) into the cell (Smith, 1963a; Smith & Hamilton-Miller, 1963; Hamilton-Miller, 1965; Ayliffe, 1965; Datta & Kontomichalou, 1965; Sabath, Jago & Abraham, 1965). This barrier seems to have some connection with resistance of *K. aerogenes* strains to penicillins, as is evidenced by the following findings: (i) in *K. aerogenes* 418 there was a relation between antibacterial activity and ease of permeation for seven penicillins (Hamilton-Miller, 1963a); (ii) 6-aminopenicillanic acid and cephaloridine, both of which obtain free access into many coliform organisms, are consistently among the most active of the β-lactam antibiotics against *K. aerogenes* (Smith & Hamilton-Miller, 1963; Hamilton-Miller, 1963a, 1965; Hamilton-Miller & others, 1965); (iii) in twelve *Klebsiella* strains a significant relation was found between intrinsic resistance and lack of permeability to benzylpenicillin (Hamilton-Miller, 1965). Izaki & others (1966) showed that a cell-free system synthesizing mucopeptide was almost as sensitive to methicillin as to benzylpenicillin whereas the intact cells were insensitive to methicillin. They concluded that these results supported the hypothesis that impermeability can play a significant role in intrinsic resistance.

There are good precedents for a lack of permeability being responsible, at least in part, for bacterial resistance to other antimicrobial agents; resistance to tetracycline (Izaki & Arima, 1963), chloramphenicol (Okamoto & Mizuno, 1964) and actinomycin D (Leive, 1965) has been explained in these terms though further investigations into the chloramphenicol-resistant bacteria revealed that resistance was due to drug destruction in some cases (Okamoto & Suzuki, 1965).

The increased incidence of hospital-acquired infections of Gram-negative origin (Kessner & Lepper, 1967) is disturbing, because such infections (notably Pseudomonas) are notoriously difficult to eradicate. The recently observed worldwide increase in the frequency of isolation of members of the Enterobacteriaceae which possess an R-factor

(R^+ strains), and thus display multiple and transferable drug resistance (see Watanabe, 1963, 1967) has aggravated the situation. Thus, Mitsuhashi, Hashimoto & others (1967) observed that most *Shigella*, *E. coli*, *Klebsiella* and *Proteus* strains isolated from clinical sources in Japan during 1965 were R^+, and H. W. Smith (1966) has demonstrated a striking increase in the isolation rate of R^+ *E. coli* strains from diseased animals, especially pigs, over the first half of the present decade.

The means by which R-factors confer resistance to β-lactam drugs are not yet clear, although there are several significant points. It is interesting to note the superficial similarity between R^+ strains and the strains with multiple resistance selected by *in vitro* training to resistance to a single drug by Sutherland & others (1964). Strains carrying an R-factor which confers resistance to ampicillin (i) produce a penicillinase which is characteristic of the R-factor, and not of the host, and (ii) possess permeability barriers to benzylpenicillin and ampicillin (Datta & Kontomichalou, 1965; Datta, 1965). Hamilton-Miller (1965, unpublished observations) has investigated the extent to which the resistance to β-lactam antibiotics shown by R^+ strains is due to penicillinase, and to what extent it is connected with manifestations of impermeability. The four strains used (very kindly given by Dr. Naomi Datta of the Postgraduate Medical School), were host *E. coli* K12 (F^-met^-), and this host carrying, singly, the three R-factors R_{1818}, R_{7268} and R_{TEM} (the subscript reference numbers signify the host strains from which the particular R-factors were isolated). Some relevant details of these strains, as determined for this experiment, are given in Table 4. The presence of an R-factor can be seen not to affect the permeability of any strain to cephaloridine,

Table 4. *Hydrolytic activities and permeability of* E. coli *K12 (host), and the same strains carrying 3 different "R" factors, toward benzylpenicillin, ampicillin and cephaloridine*

	Benzylpenicillin		Ampicillin		Cephaloridine	
	*Activity	†PF	*Activity	†PF	*Activity	†PF
E. coli K12	1·5	1	0·15	1	2·7	1
Host + R_{1818}	18·4	3	36·8	3	10·3	1
Host + R_{7268}	200	8	380	8	158	1
Host + R_{TEM}	1422	9	2262	11	1280	1

* Activity = Rate of hydrolysis (nmol substrate destroyed/min) at pH 7·4 and 37° C in presence of M/40 sodium phosphate buffer, by a suspension containing 10^9 bacteria/ml, after disruption.

† PF = Permeability Factor = $\frac{\text{rate of hydrolysis by disrupted suspension}}{\text{rate of hydrolysis by same suspension, intact}}$

See Hamilton-Miller (1965) for full experimental details.
Results for *E. coli* K12 (host) have been published already (Hamilton-Miller & others, 1965).

Table 5. *Minimum inhibitory concentrations (μg/ml) of 3-β-lactam antibiotics against large (10^6) and small (10) inocula of* E. coli *K12 (host), and the same strain carrying 3 different "R" factors*

	Benzylpenicillin			Ampicillin			Cephaloridine		
	10^6	10		10^6	10		10^6	10	
	MIC			MIC			MIC		
E. coli	62	31	(2)	20	2·5	(8)	16	2	(8)
host + R_{1818}	500	31	(16)	1000	31	(32)	31	2	(16)
host + R_{7268}	4000	125	(32)	4000	250	(16)	125	4	(32)
host + R_{TEM}	8000	1000	(8)	8000	1000	(8)	500	8	(64)

Figures in brackets, MIC for large inoculum ÷ MIC for small inoculum, represent amount of "penicillinase"-type resistance.

whereas with the two penicillins, the higher the penicillinase activity, the larger the permeability factor. Minimum inhibitory concentrations of the three antibiotics against large and small inocula of the four strains were then measured, by the tube dilution technique. Table 5 shows that, with cephaloridine, there is little or no increase in intrinsic resistance conferred by the presence of an R-factor, and that rises in hydrolytic activity go along with increases in "penicillinase-type" resistance. For the two penicillins, on the other hand, there are definite rises in intrinsic resistance, and no relation exists between increases in "penicillinase-type" resistance and hydrolytic activity. Thus it seems likely that, while an increase in penicillinase activity can account for the increased resistance to cephaloridine, alone, it cannot explain the much greater increase in resistance to benzylpenicillin and ampicillin. These latter increases are paralleled closely by rises in permeability factors, and it may well be that the fact that R^+ strains are not freely permeable to benzylpenicillin and ampicillin at least partly explains the increase in resistance to these two compounds. It seems, on the evidence at present available, that R^+ strains are best classified in Class IV.

Studies at the Pasteur Institute (Chabbert & Baudens, 1966; Baudens & Chabbert, 1967) have shown that there is no relation between the level of resistance to penicillins and the amount of penicillinase activity, in naturally-occurring R^+ salmonellae; such a relation would be expected if there were a "cause and effect" between resistance and penicillinase (Hamilton-Miller, 1965). Decreases in cellular permeability to drugs have also been proposed by other workers to account for R-factor mediated resistance (D. H. Smith, 1966; Unowsky & Rachmeler, 1966). Of particular interest in this context are the findings of Guinee & others (1967) who found that infection of *Salm. panama* with an R-factor results in a change in phage-type of the infected strain. This may be indicative of a radical alteration in the surface structure of the cells, which could alter permeability.

Transferable multiple resistance poses a threat of extreme potential danger; outbreaks of infections caused by R^+ strains can spread quickly and are often difficult to treat (Lewis, 1967; Anderson, 1968). Yoshikawa & Sevag (1967), however, report that R^+ strains of *E. coli* are sensitive to mepacrine, while R^- strains are not; they deduce that R-factors may confer sensitivity to mepacrine; it is conceivable that this finding may be of clinical significance.

CONCLUSIONS

Bacterial resistance to β-lactam antibiotics can result from two separate mechanisms, drug inactivation and drug tolerance, either or both of which may be responsible for the overall resistance exhibited by the organism in question. As a result, three classes of bacterial resistance can be observed.

Although laboratory studies of bacteria selected for increased resistance to β-lactam drugs usually result in the isolation of mutants which are resistant due to drug tolerance the validity of such investigations is doubtful as it is rare for this to be the sole mechanism of resistance noted clinically. Two species, however, do show this type of resistance *in vivo*, the gonococcus and *Strep. viridans*. With bacteria like these it would seem that *in vitro* studies have some meaning. Thus drug tolerance can be a danger since it is relatively easy to select increasingly resistant organisms during serial subculture in increasing concentration of β-lactam drugs.

The most important organism causing serious clinical resistance by β-lactam drug inactivation has been the penicillinase-producing *Staph. aureus*. It is significant that

the penicillinase of this species is an inducible enzyme and thus cultures are resistant because much higher levels of penicillinase are stimulated by the presence of a β-lactam drug. Fortunately, single cocci are little more resistant than members of the same species which lack penicillinase, probably because they succumb to penicillins before enzyme induction can occur. As a consequence, when the penicillinase-stable penicillins were introduced they had and are continuing to have success in overcoming the resistance of the penicillinase-producing *Staph. aureus.*

The same approach towards resistance by drug inactivation cannot be taken with those Gram-negative bacteria where penicillinase production is constitutive (i.e. non-inducible). If the cells produce sufficient enzyme, individual bacteria may be resistant owing to the penicillinase they possess, but if the level of production per cell is low, single cells may not be significantly resistant. However, large numbers of such cells may exhibit therapeutic resistance which may be of a low degree since a modest increase in penicillin concentration may inhibit growth; nevertheless this increase may be outside the scope of therapeutic blood levels.

Generally these two cases may themselves be oversimplified since inherent resistance is additionally often present in penicillinase-producing Gram-negative bacteria. In many cases such organisms are sufficiently resistant by the non-destructive mechanism that penicillinase production appears superfluous. Another similar example is the production of penicillinase by type I *E. coli.* When mutants more highly resistant to ampicillin are selected these contain more penicillinase than the parent strain and yet the extra penicillinase produced seems of little avail since it does not hydrolyse ampicillin significantly.

This raises the question of the real function of penicillinase (Pollock, 1967). It is uncertain whether any of the β-lactam antibiotics are the substrate or the analogues of a hitherto undiscovered substrate, the enzymic alteration of which is useful for bacterial metabolism rather than primarily a defensive mechanism against antibiotic attack.

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