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Historical perspective

Forty years of β-lactam research

George N. Rolinson*

Parkgate House, Newdigate, Surrey RH5 5DZ, UK

From the Editor-in-Chief: For more than 40 years the author has been involved in research in the field of β-lactam antibiotics. Much of this work was concerned with the development of the semisynthetic penicillins, following the isolation of the penicillin nucleus, 6-aminopenicillanic acid. This work resulted in the introduction of a number of important antibiotics including methicillin, cloxacillin, flucloxacillin, ampicillin, amoxycillin, carbenicillin and ticarcillin. Many of these compounds also provided the incentive or the basis for studies of a more fundamental nature in cell biology and antibacterial chemotherapy. The following is the author's personal account of some of the studies in the β-lactam field in which he has been involved.

Introduction

The history of the β-lactam antibiotics is generally considered to begin with Alexander Fleming and his observation in 1928 that a strain of the mould *Penicillium* produced a diffusible antibacterial agent which he named penicillin.[1] Fleming carried out a number of studies on the antibacterial activity of penicillin *in vitro* using the filtrate from liquid cultures of *Penicillium*. He also showed that the culture filtrate appeared to be non-toxic when injected into mice and rabbits. However, he did not carry out any studies of penicillin against experimental infections in animals, and thus failed to demonstrate an essential property of penicillin, namely its ability to overcome bacterial infection when administered systemically.

It may seem surprising that Fleming did not investigate the chemotherapeutic properties of penicillin *in vivo*, since such experiments would be commonplace today, but it must be remembered that, at the time of Fleming's observation, the age of antibiotics had not begun. The sulphonamides had yet to appear and, with the exception of Ehrlich's Salvarsan, no systemic antimicrobial agents were known at that time. Consequently penicillin was considered for clinical use only as an antiseptic for local application.

Attempts to isolate and purify penicillin in the 1930s were largely unsuccessful and by the end of the 1930s interest in penicillin had almost disappeared. Interest was revived, however, in a dramatic way in May 1940 when Florey and Chain and their colleagues at Oxford showed that penicillin injected subcutaneously was highly effective against a lethal streptococcal infection in mice.[2] It was this demonstration of the systemic chemotherapeutic activity of penicillin that provided the stimulus for the development of penicillin and its clinical use in humans.

The introduction of penicillin into clinical practice itself provided the stimulus for the discovery in the 1940s and 1950s of many other antibiotics including streptomycin, chloramphenicol, chlortetracycline, erythromycin, vancomycin and kanamycin. During this period the β-lactam field, in clinical terms, comprised only two compounds, namely penicillin G and penicillin V, and the major expansion of the β-lactam field only occurred in the early 1960s with the development of the semisynthetic penicillins, quickly followed by the development of semisynthetic cephalosporins and other β-lactam antibiotics. However, before this development, an important area of microbiological work in the 1940s and 1950s was the fermentation process responsible for the production of penicillin. This was the subject of my early studies in the β-lactam field.

*Formerly Associate Director of Research and Senior Microbiologist, Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey.

Penicillin fermentation studies, 1952–1954

Work on the penicillin fermentation included studies on the utilization of fats and carbohydrates during the fermentation process and the relationship between penicillin production and aeration.[3,4] In the course of this work it became clear that it would be helpful if penicillin production could be studied under defined conditions, which remained essentially constant over the period of the experiment, in contrast to the complex changes which occurred in conventional studies at that time of fermentations extending over a period of 3–5 days. Accordingly a procedure was introduced[5] in which samples of mycelium from penicillin fermentations were washed free from penicillin and resuspended in media of known composition. The suspensions were then incubated under shaking conditions and the penicillin assayed after a period of 1–1.5 h. Penicillin production could then be expressed as micrograms of penicillin produced/mg dry mycelium/h (Q_{pen}). These were the first experiments in which penicillin production was studied in this way.

The mycelial suspension was incubated in Warburg manometer flasks, thus permitting simultaneous measurement of oxygen uptake. Aeration conditions and the resulting availability of dissolved oxygen to the mycelium could be varied depending on the density of the mycelial suspension, the volume of suspension per flask and the rate of agitation. Using this technique it was found that maximum penicillin production occurred only if aeration conditions were such that the mycelium was able to respire at the maximum rate. If aeration conditions limited the rate of oxygen uptake to half of the maximum then the rate of penicillin production was correspondingly halved. However, penicillin production was not simply proportional to oxygen uptake *per se*. This could be shown when mycelium was suspended in media of different nutritional composition. Under such conditions the rate of oxygen uptake varied considerably, as would be expected, but penicillin production was independent of the absolute rate of respiration provided oxygen uptake was not limited by the conditions of aeration.

Penicillin production by suspensions of washed mycelium continued at a uniform rate for at least 3 h and over this period of time penicillin production was found to be independent of the nutritional composition of the medium, the rate of production being the same when the mycelium was suspended in a salts/phenylacetate solution as it was in a complete corn steep/lactose fermentation medium. Contrary to the understanding at the time, it was also found that the inclusion of glucose in the suspending medium did not have an inhibitory effect on penicillin production provided that the increased oxygen demand created by this substrate could be satisfied by the conditions of aeration. If this was not the case then the rate of penicillin production was diminished, but if adequate aeration could be provided to meet the increased oxygen demand, then penicillin production was not depressed by glucose. These results provided an understanding of the need to maintain glucose concentrations at a low level by a continuous feed when this particular sugar was used in penicillin fermentations.[6]

Experiments using the washed mycelium technique also showed that penicillin biosynthesis was extremely sensitive to cyanide.[5] At concentrations of cyanide that had a negligible effect on respiration penicillin formation was almost completely inhibited. With hindsight it is tempting to speculate that the system inhibited by cyanide in these experiments was the iron-dependent dehydrogenase now known to be responsible for ring closure of the Arnstein tripeptide in penicillin biosynthesis.

The penicillin nucleus, 6-APA, 1956–1957

Towards the end of 1955, I joined what was then Beecham Research Laboratories to set up microbiological research at the laboratories at Brockham Park in Surrey. At the same time Ernst (later Sir Ernst) Chain was engaged as a consultant. He felt that penicillins with new and useful properties might be obtained if the acyl side-chain of the penicillin molecule could be varied more extensively than was possible by the use of precursors in the fermentation medium. It was his suggestion that a penicillin be prepared by fermentation that could subsequently be modified by chemical means. The penicillin chosen as the starting point for such chemical modification was *p*-aminobenzyl penicillin. It had already been reported that *p*-aminobenzyl penicillin could be obtained by fermentation using *p*-aminophenylacetic acid as precursor[7] but no details of the antibacterial potency of this penicillin had been provided. Accordingly, in our own fermentation work on the preparation of *p*-aminobenzyl penicillin, I decided to use two independent assay procedures. One method was the cup-plate assay, in which antibacterial activity is measured in comparison with a standard solution of penicillin; the other was a chemical method involving reaction of the β-lactam ring of the penicillin molecule with hydroxylamine and the colorimetric assay of the resulting hydroxamic acid by the addition of ferric salt. In fermentations in which *p*-aminophenylacetic acid was added as precursor it was found that the two assay methods gave very similar results. On the other hand, in control fermentations in which no precursor was added, the two assays showed a wide discrepancy, the chemical assay giving a value very much higher than the assay for antibacterial activity. From these results it seemed likely that, under fermentation conditions in which there was a lack of available side-chain structures, the nucleus of the penicillin molecule was produced without any side-chain attached (Figure 1). Such a compound could be expected to be assayed by the chemical method but might well show little antibacterial

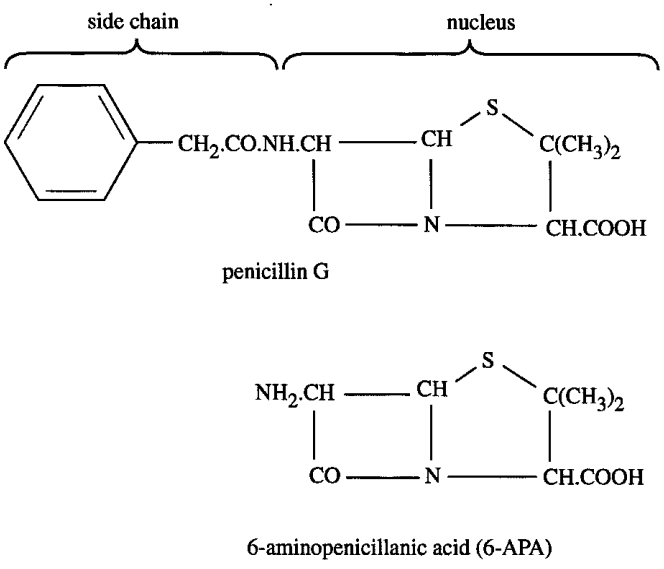

Figure 1. Structure of penicillin and 6-aminopenicillanic acid.

activity. This interpretation proved to be correct and my colleagues and I[8] showed the compound to be the penicillin nucleus, 6-aminopenicillanic acid (6-APA). This compound made possible the development of the semisynthetic penicillins because new side-chain structures could readily be attached by chemical means through the free amino group.

Semisynthetic penicillins introduced by Beecham Research Laboratories in the early 1960s included methicillin (1960), ampicillin (1961) and cloxacillin (1962). At about the same time the structure of the naturally occurring cephalosporin, cephalosporin C, was established by Abraham & Newton.[9] Removal of the side-chain of cephalosporin C provided a nucleus, 7-ACA, from which semisynthetic cephalosporins could be prepared in a manner analogous to the development of the semisynthetic penicillins.[10] However it is significant that the cephalosporin nucleus, 7-ACA, unlike the penicillin nucleus, 6-APA, is not a naturally occurring substance and can only be obtained by the removal of the side-chain from cephalosporin C. The question arises, therefore, as to whether there would have been the incentive to attempt this side-chain removal had it not been for the discovery of the penicillin nucleus and the demonstration that important semisynthetic derivatives could be prepared from it. In this connection it should be remembered that before the isolation of 6-APA no attempt had been made to remove the side-chain from the penicillin molecule in order to obtain a starting point for the preparation of semisynthetic derivatives. It may be, therefore, that had it not been for the discovery and isolation of the penicillin nucleus the development of the cephalosporins would not have taken place.

Production of 6-APA by enzymatic hydrolysis of penicillin, 1959–1960

The importance of 6-APA as a starting point for the preparation of semisynthetic penicillins provided a strong stimulus for research into the production of this compound. Although 6-APA was first obtained as a naturally occurring fermentation product, the yields were always considerably lower than those of penicillin G or penicillin V obtained in fermentations in which the appropriate precursor was used. Moreover, the isolation of 6-APA from the fermentation broth was not easy. It seemed possible that an alternative route to 6-APA might be the enzymatic removal of the side-chain of the penicillin molecule. Examination of various microorganisms in our laboratories revealed that at least two types of penicillin deacylase occurred in nature.[11] One type was found to be widely distributed among the actinomycetes and filamentous fungi. This type of enzyme hydrolysed penicillin V readily but penicillin G was hydrolysed only slowly. The other type of deacylase was detected among bacteria; it hydrolysed penicillin G very readily but penicillin V was split only at about 20% of the rate.

A process for the manufacture of 6-APA by deacylation of penicillin was first developed in our laboratories using a deacylase obtained from *Streptomyces lavendulae* with penicillin V as substrate; a patent for this process was filed in March 1959. Subsequently the deacylase of bacterial origin was discovered independently in a number of laboratories[11–14] and a process for 6-APA production was then developed using this type of enzyme with penicillin G as substrate.

Semisynthetic penicillins, 1959–1970

With the availability of 6-APA and the opportunity to prepare semisynthetic penicillins, a number of objectives presented themselves. These included the development of penicillins showing improved oral absorption and the preparation of penicillins with a broader spectrum of activity than that shown by penicillins G or V. Another objective was the development of penicillins stable to staphylococcal β-lactamase and active against the penicillin-resistant strains of *Staphylococcus aureus* which had emerged as a serious clinical problem. These objectives were all realized within a few years of the isolation of 6-APA. The first semisynthetic penicillin to be introduced into clinical practice was a close analogue of penicillin V, namely phenethicillin. This was followed by ampicillin, methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin, flucloxacillin, carbenicillin, amoxycillin and ticarcillin (Figure 2). The microbiological evaluation of these penicillins was directed by the author together with studies on absorption and excretion in volunteers following oral and or parenteral administration. The properties of these

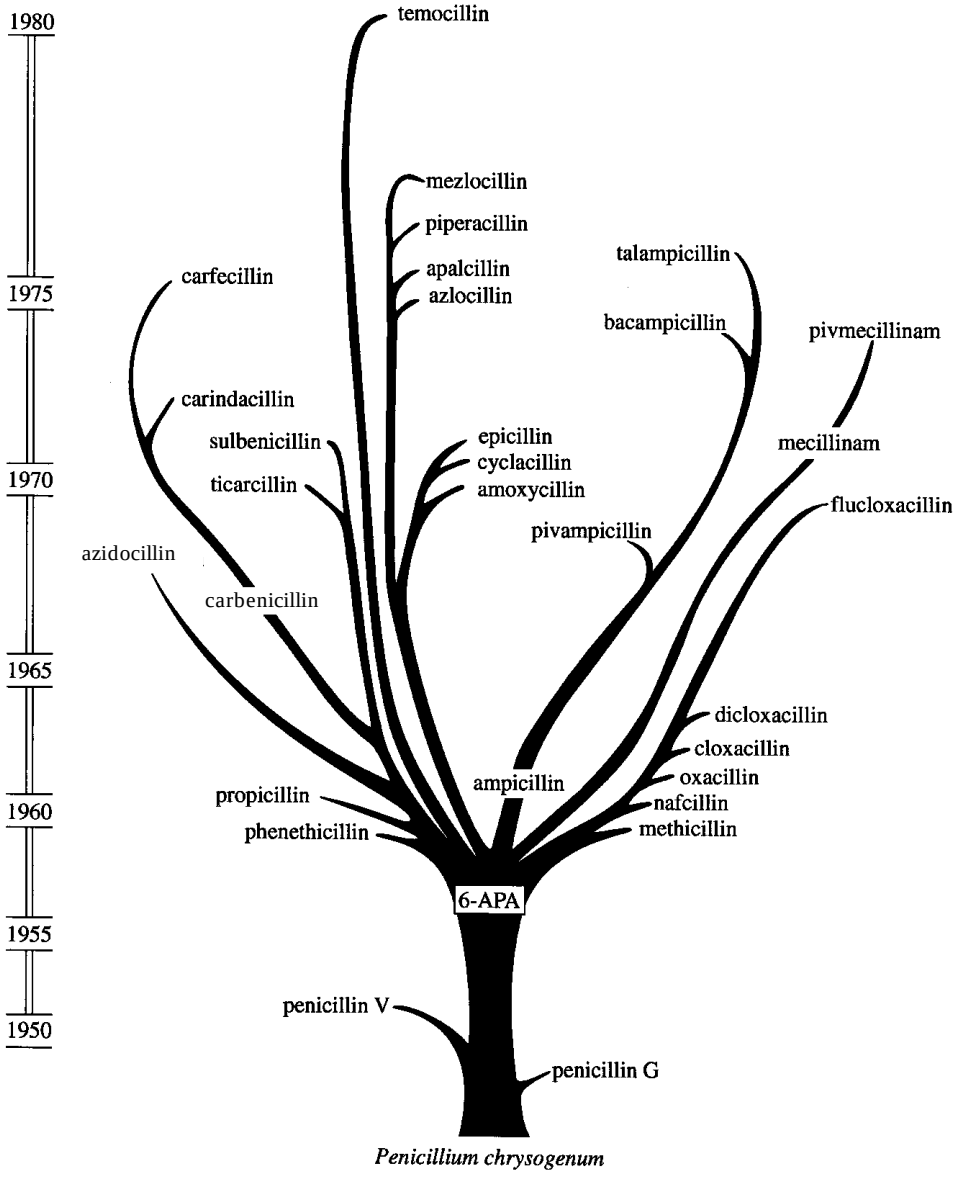

Figure 2. Development of the semisynthetic penicillins.

semisynthetic penicillins and other β-lactam antibiotics were reviewed by the author in 1973 and again in 1986.[15,16]

Methicillin and MRSA, 1960–1963

The first semisynthetic penicillin stable to staphylococcal β-lactamase and clinically effective against penicillin-resistant *S. aureus* was methicillin. In the original publication on the microbiology of methicillin[17] the strains of *S. aureus* tested were found to be uniformly susceptible. However, shortly after the introduction of methicillin into clinical practice three strains showing diminished susceptibility were encountered by Patricia Jevons at the Central Public Health Laboratory at Colindale.[18] My examination of these strains[19] showed them to be heterogeneous in their resistance to methicillin. This could be seen when large numbers of cells were inoculated on to agar containing doubling dilutions of methicillin. After incubation overnight at 37°C, the majority of the population was found to show normal susceptibility and only a small minority proved resistant. This latter fraction was itself heterogeneous with respect to the level of resistance. At a concentration of 12.5 mg/L, one in 10^7 of the population was able to grow but only one in 10^8 were resistant at a concentration of 250 mg/L. These findings were later reported in more detail.[20] In this study seven strains of methicillin-resistant *S. aureus* (MRSA) were examined together with a typical methicillin-susceptible strain (Table I). Large numbers of cells were again inoculated on

Table I. Determination of proportion of methicillin-resistant cells in seven strains of methicillin-resistant *S. aureus*[a] (data from Sutherland & Rolinson[20])

	Methicillin concentration (mg/L)							
Strain	500	250	125	50	25	12.5	5.0	2.5
13137	0	0.1	0.8	0.6	0.6	2	5×10^5	10^6
QMH 1	0	0.75	1.3	4	35	25	7×10^5	10^6
8054	2.5	6	6	45	100	250	10^6	10^6
5974	1	10	4	5	6	35	5×10^4	10^6
BRL 1776	0.3	0.5	0.8	1	1.2	6	30	10^6
BRL 1800	0	0.1	0.3	2	2	5	–	10^6
1244/62	1	3	3	6	40	50	10^6	10^6
BRL 1089[b]	0	0	0	0	0	0	0.5	10^6

[a]Results expressed as the number of colonies/10^6 plated that grew at indicated concentration of methicillin after 48 h incubation at 37°C.
[b]Typical methicillin-susceptible, β-lactamase-positive *S. aureus*.

to agar containing doubling dilutions of methicillin. With the methicillin-susceptible strain only one in 2×10^6 of the population formed colonies at a concentration of 5 mg/L and no colonies developed at 12.5 mg/L. The seven strains of MRSA were all heterogeneous in their resistance to methicillin, the proportion of the cultures showing diminished susceptibility ranging from 2 to 250 per 10^6 of the population at 12.5 mg/L methicillin, 0.6 to 45 per 10^6 at 50 mg/L and 0.1 to 10 per 10^6 at a concentration of 250 mg/L. The cells exhibiting resistance grew more slowly than normal cells both in the presence and in the absence of methicillin.

When resistant colonies from MRSA strains were subcultured from plates containing high concentrations of methicillin, the resulting cultures were found to be uniformly resistant, the entire population of cells being capable of forming colonies at concentrations of methicillin as high as 500 mg/L. However, after six transfers in drug-free medium these cultures had reverted to heterogeneous populations of cells in which the majority were susceptible with only a minority showing resistance.

It is now recognized that the mechanism of resistance in MRSA is the production of an extra penicillin-binding protein (PBP2a) which has low affinity for methicillin and other β-lactam antibiotics.[21,22] It also seems likely that the genetic determinant for the production of this extra PBP is present in all the cells of a population of an MRSA culture. Although it is now more than 30 years since the phenomenon of heterogeneity in MRSA was first described,[19] it is still not clear why the resistance to methicillin is expressed only in a small proportion of the population of cells when incubated at 37°C nor is it clear why the cells grow more slowly when expressing resistance but revert to a normal rate of growth when the expression of this character is lost.

Studies on staphylococcal β-lactamase, 1961–1963

The development of semisynthetic penicillins stable to staphylococcal β-lactamase and clinically effective against penicillin-resistant staphylococci provided the incentive for studies, with colleagues, on the nature of the β-lactamase produced by *S. aureus*.[23] These studies included work on the cellular location of staphylococcal β-lactamase and on the preparation of cell-free enzyme. Studies were also carried out on the kinetic properties of the enzyme. The finding that the K_m with benzyl penicillin as substrate varied depending on the strain of *S. aureus* used was one of the earliest indications that different types of β-lactamase are produced by staphylococci. Methicillin also provided a valuable tool in studies on β-lactamase induction as a result of its high degree of stability to β-lactamase. Studies were also carried out at this time on the inactivation of staphylococcal β-lactamase by methicillin and other penicillins.

Ampicillin and the role of β-lactamase in resistance, 1961–1964

One objective in preparing semisynthetic penicillins from the 6-APA nucleus was to develop penicillins with a broader spectrum of antibacterial activity than that shown by penicillins G or V. The first penicillin of this type to be introduced into clinical practice was ampicillin. This penicillin showed increased activity against a number of Gram-negative pathogens but it was recognized that strains of these organisms were not uniformly susceptible. In the original report on the microbiology of ampicillin,[24] data were presented on β-lactamase production in relation to resistance to ampicillin among Gram-negative bacilli. In

these experiments residual ampicillin was determined in broth MIC tests after incubation overnight at 37°C. With some cultures, resistance to ampicillin appeared to correlate closely with inactivation of the drug. With one strain of *Escherichia coli*, however, this appeared not to be the case. The MIC for this culture was 250 mg/L and, although some inactivation of the drug occurred, the residual level of ampicillin after overnight incubation remained as high as 100 mg/L. It was concluded that an intrinsic mechanism of resistance to ampicillin was present in this culture. This conclusion may have been correct but knowledge over the last 30 years has shown that failure to bring about inactivation of ampicillin in the culture medium surrounding the bacterial cell does not necessarily eliminate β-lactamase as the basis of resistance. We now know that β-lactamase located in the periplasmic space may result in a high level of resistance without causing a marked reduction in the concentration of antibiotic external to the cell.

The activity of ampicillin against clinical isolates of Gram-negative bacilli was the subject of a further publication in 1964.[25] It was concluded that ampicillin resistance in some isolates was due to β-lactamase production and in other cases to intrinsic mechanisms of resistance. Some isolates appeared to show both characteristics. Moreover β-lactamase production did not always result in a high level of resistance. These findings together with those of other workers, notably Ayliffe,[26] Percival *et al.*[27] and Smith & Hamilton-Miller[28] were among the earliest investigations into the role of β-lactamase in ampicillin-resistance among Gram-negative bacilli.

The isoxazolyl penicillins and the significance of protein binding, 1962–1983

The isoxazolyl penicillins, oxacillin and cloxacillin, made their appearance in 1962, followed a few years later by two further members of this series, namely dicloxacillin and flucloxacillin. These penicillins proved to be more active than methicillin against penicillin-resistant staphylococci and also had the advantage of being absorbed when given by mouth. However, unlike methicillin, the isoxazolyl penicillins showed a high level of protein binding. As a result of this binding the activity of the isoxazolyl penicillins was markedly diminished in the presence of serum, and in blood level assays the use of standards prepared in human serum was essential if valid results were to be obtained.

When the isoxazolyl penicillins were introduced in the early 1960s, the basic principles of protein binding had already been established. It was known that many antibiotics, including penicillins, could combine reversibly with the proteins in serum and other body fluids and tissues resulting in an equilibrium between protein-bound drug on the one hand and free antibiotic on the other and it was known that antibiotic molecules bound to plasma proteins were essentially devoid of antibacterial activity. The effect of protein binding on the distribution of antibiotic between vascular and nonvascular compartments had also been established. Despite this basic knowledge, the overall significance of protein binding as a factor in antibacterial chemotherapy was not widely understood in the 1960s. Reference to the published work bears witness to the uncertainty that prevailed in many quarters at that time. For example, Barber & Garrod[29] expressed the view that "the extent to which plasma binding interferes with therapeutic efficacy remains to be determined" while Bond *et al*.[30] wrote that "the relative importance of bound and dissociated penicillin is arguable". Klein & Finland[31] commented that "the binding of the new penicillins to serum proteins has been studied but the clinical significance remains unclear" and similar uncertainty regarding the significance of protein binding was expressed by other workers including Geraci *et al.*[32] and Kirby *et al.*[33]

Against this background of uncertainty, I sought to bring together the various aspects of protein binding in a single unified account which would allow the significance of protein binding in antibacterial chemotherapy to be understood more clearly. This was first presented at a symposium held in London[34] and a more detailed review was published later.[35] These accounts sought to show that, although protein binding is readily reversible, the effect is to confine antibiotic molecules within the vascular compartment in a non-diffusible form and in this way limit the concentration of free and antibacterially active drug that can be reached in the tissues. A similar understanding was expressed by other authors, notably Kunin,[36] Craig & Kunin[37] and Peterson & Gerding[38] before a restatement was presented by me in 1980.[39] These views on the significance of protein binding increasingly gained acceptance but direct experimental evidence of the effect of protein binding on therapeutic efficacy *in vivo* was not available until 1983 when my colleagues and I published the results of a study using an experimental infection in mice.[40] In this study a group of seven isoxazolyl penicillins were chosen which all showed essentially the same level of antibacterial activity *in vitro* (in the absence of serum) but differed in their extent of binding in mouse serum, the values ranging from 36% to 98%. Therapeutic activity (CD_{50}) was shown to be directly related to the extent of binding in serum.

It is not unusual to find that phenomena encountered in recent years have their explanation in findings reported at a much earlier time and this is true in the field of protein binding. In a study on the basic aspects of protein binding of antibiotics, Rolinson & Sutherland[41] showed that the extent of binding in human serum is largely independent of the concentration of antibiotic until a level of approximately 200 mg/L is reached. For most β-lactam antibiotics this corresponds to approximately one molecule of antibiotic per molecule of plasma protein and under these circumstances the available binding sites are largely

occupied. Consequently with further increase in antibiotic concentration the proportion of unbound drug increases markedly. This is the basis of the so-called dose-dependent pharmacokinetics seen with certain antibiotics. This phenomenon is not strictly a property of the antibiotic as such but results from a change in the proportion of free to bound drug when certain antibiotics are administered in a dosage that results in serum levels in excess of approximately 200 mg/L.

Serum concentrations following administration of penicillins, 1959–1972

Studies on the serum concentrations of phenethicillin, ampicillin, cloxacillin, flucloxacillin and amoxycillin following oral administration to volunteers were carried out as part of the evaluation of these penicillins. The results provided a basis for the choice of dosage in the clinical usage of these agents but these studies also provided data on some fundamental aspects of penicillin absorption following oral administration. For example, penicillins that are closely related in chemical structure may nevertheless differ markedly in the extent of absorption when taken by mouth. This is illustrated by the results with amoxycillin and ampicillin. These penicillins are closely related in their structure and in their chemical and physical properties yet serum concentrations of amoxycillin are approximately twice those obtained with the same dose of ampicillin.[42] This difference cannot be accounted for on the basis of the relative stability of these compounds or the result of differences in aqueous or lipid solubility. The pK_a values for the two compounds are also very similar and do not provide an explanation for the difference in absorption.[43] The reason for the superior absorption of amoxycillin remains unclear.

Another aspect of absorption following oral administration is the variation in the serum levels reached within a group of subjects. With certain penicillins absorption appears highly uniform among individuals, but with other penicillins there is considerable variation in the peak levels reached. For example, in the original study on the absorption of ampicillin in volunteers,[44] the peak levels following a 250 mg dose varied among individuals from 1.3 to 3.5 mg/L, a range of less than three-fold. With a dose of 1 g the peak levels varied from 4.5 to 8.5 mg/L, a range of less than two-fold. On the other hand, in a study carried out with cloxacillin,[45] peak serum levels varied from 1.5 to 30 mg/L, a 20-fold difference. This range was not the result of isolated values; approximately 30% of the subjects had peak levels either <4 mg/L or >18 mg/L, the mean value for the group as a whole being 9.6 mg/L.

In this study marked variation could also be seen in the peak levels reached in individual volunteers tested on different occasions, despite administration of the drug in the fasting state and at the same time of day. For example, one volunteer showed a peak cloxacillin concentration of 12.5 mg/L on one occasion and 46 mg/L on the second occasion. Another volunteer showed a concentration of 20 mg/L on the first occasion and only 3.9 mg/L on the second. The reason for variation in blood concentrations with penicillins such as cloxacillin has not been established but it is clear that it cannot be accounted for simply on the basis of good absorbers and bad absorbers.

β-Lactams active against *Pseudomonas aeruginosa*, 1967–1981

The first β-lactam antibiotic to show clinically useful activity against *P. aeruginosa* was carbenicillin.[46] When this penicillin was introduced in 1967, the choice of agents available for the treatment of infections caused by *P. aeruginosa* was severely limited. Gentamicin had only recently been introduced and none of the penicillins and cephalosporins in clinical use at that time were active. Latamoxef, imipenem and the cephalosporins active against *P. aeruginosa* did not appear until the late 1970s and early 1980s. The introduction of carbenicillin in 1967, therefore, provided an important addition to the agents available for the treatment of *P. aeruginosa* infections.

Although carbenicillin was clearly active against *P. aeruginosa*, the original evaluation showed that, in terms of MIC, its activity was not high.[46] Most strains required a concentration of carbenicillin as high as 50 mg/L for inhibition. However, the compound showed low toxicity and could be administered in high dosage.

Early studies showed carbenicillin to be effective in the treatment of *P. aeruginosa* infections[47,48] and the clinical place of this penicillin was soon established. Later the thiophene analogue of carbenicillin, namely ticarcillin, was introduced.[49] This penicillin showed essentially the same spectrum of activity as carbenicillin but was significantly more active against *P. aeruginosa*.

Carbenicillin and ticarcillin were both shown to be bactericidal against *P. aeruginosa*, as would be expected of β-lactam antibiotics. However, the kill curves showed that, after an initial period of bactericidal action in the presence of carbenicillin, the viable count increased and this regrowth could reach visible proportions after incubation overnight.[50] When this growth was used as inoculum in repeat tests the same pattern of bactericidal action followed by regrowth was observed. The phenomenon of regrowth, therefore, did not appear to be the result of emergence of a resistant fraction of the population and it was also shown that it was not the result of inactivation of the antibiotic.[50]

The regrowth of *P. aeruginosa* after exposure to carbenicillin was investigated in a later study in our laboratories.[51] In cultures showing this phenomenon a ring of growth could be seen adhering to the vessel wall at the junction with the surface of the culture medium. In

addition some growth was often visible as a surface pellicle. Microscopic examination of the bacterial cells freely suspended in the medium showed them to be chiefly in the form of long filaments, while the growth adhering to the vessel wall consisted largely of densely packed cells of normal morphology. Microscopic preparations flooded with Indian ink[52] revealed an area surrounding these aggregations of cells which was consistent with polysaccharide capsular material. The cells of *P. aeruginosa* adhering to the vessel wall were markedly less susceptible to carbenicillin than the cells freely suspended in the culture medium.[51] It was concluded that adhesion to the vessel wall permits bacterial growth to develop relatively uninfluenced by the presence of the antibiotic and that fragmentation of this growth then allows normal cells to be introduced continually into the medium resulting in the observed increase in viable count.

The importance of bacterial growth in the form of biofilms is now well recognized though the reason for the diminished antibiotic susceptibility of such cells is not well established. It may be that the glycocalyx which typically envelops the cells in a biofilm presents a barrier to the diffusion of antibiotics and nutrients. Cells in a biofilm typically show low growth rates and this itself would result in diminished susceptibility to β-lactam antibiotics.

Studies with experimental infections in animals, 1969–1975

The evaluation of individual semisynthetic penicillins by myself and my colleagues included in-vivo studies using experimental infections in animals. Results of particular interest were obtained with ampicillin and amoxycillin. These penicillins are closely related in chemical structure and show a very similar level of antibacterial activity *in vitro*. However, against intraperitoneal infections in mice, amoxycillin was significantly more active than ampicillin after oral or subcutaneous administration.[53] Further studies were carried out using an intramuscular infection in mice.[54] The animals were infected in the hind leg with approximately 10^7 viable bacteria using *E. coli* and *Proteus mirabilis* strains. The inoculation was made in the muscles of the back of the thigh as originally described by Selbie & O'Grady.[55] The thigh diameter was measured daily. Over the first 24 h following inoculation, the thigh diameter increased from the normal value of *c.*3.7 mm to >8.0 mm and the number of viable bacteria increased from 10^7 to $>10^9$ cfu over the same period. Mortality ranged from 0 to 30% after 24 h and from 40% to 100% after 48 h.

With adequate dosage both ampicillin and amoxycillin were therapeutically effective, resulting in no appreciable thigh enlargement and no mortality. However, amoxycillin proved to be effective at a significantly lower dose than ampicillin despite similar activity *in vitro*, in terms of MIC, against the infecting organisms. Moreover, following subcutaneous administration, the concentrations of ampicillin and amoxycillin in serum and in tissue homogenate were found to be very similar, yet amoxycillin was therapeutically more active than ampicillin.

This difference in activity could be correlated with the rate of bactericidal action of these penicillins. Counts of the number of viable bacteria in homogenates of the infected muscle showed that the bactericidal action of amoxycillin was more rapid and more complete than that of ampicillin when the two penicillins were administered at the same dose.[54] This difference in the rate of bactericidal action could also be seen *in vitro* and was the subject of a later study.[56]

Results of interest were also obtained with carbenicillin using an intramuscular infection with *P. aeruginosa* in mice.[57] These results illustrated the importance of the time interval between one dose of carbenicillin and the next in the treatment of this infection. The antibiotic was highly effective when administered every 2 h but the therapeutic effect diminished progressively when the interval between doses was extended beyond 2 h. It was also shown that 2 h represented the time when the blood concentration of carbenicillin had fallen below the MIC for the infecting organism. If the next dose of carbenicillin was delayed beyond this point, counts of viable bacteria in homogenates of the infected muscle indicated a prompt resumption of growth of surviving bacteria with a corresponding impairment of therapeutic effect. The lack of a post-antibiotic effect with a Gram-negative bacillus such as *P. aeruginosa* is discussed in the following section.

Studies on the effect of β-lactams on the bacterial cell, 1973–1980

The post-antibiotic effect

Shortly after penicillin was first introduced into clinical practice it was shown by Parker & Marsh[58] and by Eagle & Musselman[59] that when staphylococci and streptococci are exposed to penicillin for a period of time and the cells then transferred to a drug-free medium this is followed by a recovery period during which time the number of viable bacteria remain essentially constant before normal growth is resumed. This recovery period later became known as the post-antibiotic effect (PAE).

In the years following the original reports, numerous authors were able to confirm the PAE in experiments with staphylococci and streptococci. However, in the late 1970s, I considered the possibility that the PAE might not be a true period of bacteriostasis but might represent the period of time required for individual surviving bacteria to restore the clusters or chains of cells characteristic of staphylococci and streptococci since it is the number of cfu that is measured in the viable count, not the number of individual viable cells. This possibility was investigated by

direct microscopic observation.[60] Staphylococci were exposed to a bactericidal concentration of benzylpenicillin in broth culture for 2 h and the antibiotic then inactivated by the adding β-lactamase. A sample of the culture was then transferred on to the surface of a thin layer of agar on a slide mounted on a heated microscope stage maintained at 37°C. Incubation was continued and a photographic record of a selected field was made at intervals of time. The results showed that, in some cases, surviving viable cells were able to resume division almost immediately after inactivation of the antibiotic but other cells did indeed show a recovery period before division was resumed and this period varied from 1 to 4 h.

Although clearly demonstrable with Gram-positive cocci, a PAE is not seen with Gram-negative bacilli, following exposure to penicillins and cephalosporins. This was first shown by the author[61] in experiments with *P. aeruginosa* exposed to carbenicillin and later with isolates of *E. coli*, *Klebsiella* and *Proteus* spp. exposed to cephalothin[62] or to ampicillin.[60]

It would appear that the lack of a PAE with Gram-negative bacilli can be correlated with the effect of the antibiotic on the bacterial cell. The primary effect of penicillins and cephalosporins is inhibition of cell division. In a coccus, such as *S. aureus*, inhibition of cell division inevitably means that normal growth is prevented since growth proceeds by a process of division. When this is inhibited the cells become swollen with evidence of internal disorganization[63] and recovery from this damage requires a period of time: hence there is a PAE following the removal of the antibiotic. On the other hand, in a bacillus, such as *E. coli*, cell division may be inhibited but growth can continue in the form of non-septate filaments[64] and this is seen with many penicillins and cephalosporins. These filaments do eventually lyse as a result of a sudden rupture of the cell wall, but if the antibiotic is removed before lysis occurs such filaments are able to resume cell division[65] and give rise to a population of normal cells. This resumption of cell division occurs very promptly following the removal of the antibiotic[56] and consequently there is no appreciable PAE.

Effect of β-lactams on cell-wall synthesis

In 1974–75 my colleagues and I carried out a number of studies by microscopic observation on the effect of β-lactam antibiotics on the bacterial cell. The results were recorded on cine film including a number of time-lapse sequences, and later some of these results were published.[56] The bacterium used in these studies was a strain of *E. coli* and the antibiotics used included ampicillin, amoxycillin, carbenicillin, cephalexin and cephaloridine.

At concentrations of 1–2 × MIC, all the antibiotics inhibited cell division with continued growth resulting in cells being longer than normal. This was followed by cell lysis. The cell wall could be seen to rupture abruptly, usually at a division site, allowing the cytoplasmic membrane to bulge out forming a spheroplast. Rupture of the spheroplast then followed, due to osmotic pressure, resulting in loss of the cytoplasmic contents and lysis of the cell.

This sequence of events, namely inhibition of cell division followed by lysis, was the same for all the β-lactams studied but marked differences were seen in the time at which lysis occurred. With certain β-lactams, for example amoxycillin, the onset of lysis was seen very early, cell growth barely reaching two cell units in length before rupture of the wall occurred. With other β-lactams, for example ampicillin, a significant period of growth occurred in the form of non-septate filaments before lysis took place. With carbenicillin, and with cephalexin, an even longer period of filamentous growth occurred before the cells lysed, again as a result of a sudden rupture of the cell wall. These differences between one β-lactam and another can be correlated with their affinity for specific PBPs.

In the presence of certain penicillins and cephalosporins, at concentrations in excess of the MIC, growth of *E. coli* in the form of non-septate cells may continue for at least 2 h, resulting in filaments that are up to 20 cell units or more in length. In 1980, I published a study[66] on the rate of growth of such filamentous cells compared with the normal rate of growth in the absence of any antibiotic. *E. coli* was grown on agar on a microscope slide incubated on a heated stage and photomicrographs were taken at intervals of time. These were enlarged and the length of individual bacterial cells was measured using a map measurer. These values were then plotted against time to record the rate of growth. In the presence of cephalexin and also carbenicillin, at concentrations that were ultimately bactericidal, the growth of filamentous cells was found to proceed at a uniform exponential rate and this rate did not differ significantly from the doubling time for normal cells growing in the absence of antibiotics. It could be said, therefore, that bacterial growth was not inhibited by these antibiotics and it follows that overall cell-wall synthesis was also not inhibited to any significant extent. This might seem at variance with the understanding of the mode of action of β-lactam antibiotics. However, as discussed already, the filamentous cells which develop in the presence of various penicillins and cephalosporins do eventually lyse, as a result of a sudden rupture of the cell wall, usually at a point where cell division would normally have taken place. In such filamentous cells, therefore, inhibition of cell wall synthesis would appear to be highly localized and confined largely to the region in which there was derangement of division.

Sub-MIC concentrations and therapeutic effect

Antibacterial activity is commonly expressed in terms of the MIC as determined *in vitro*, usually after overnight

incubation. However it has long been appreciated that antibiotic concentrations below the conventional MIC may nevertheless exert a significant degree of antibacterial effect.[67–70] These effects include a prolongation of the lag phase, a reduction in the rate of growth and a diminution in the size of the bacterial population reached in the stationary phase.

The activity of sub-MIC concentrations has a bearing on the antibiotic concentrations that should preferably be reached in the blood in the treatment of infection. It is widely assumed that concentrations in the blood should at least equal the MIC and preferably exceed this value several-fold, but the basis for this is not well established. In a study carried out in 1979[71] serum concentrations of a number of penicillins were measured in mice experimentally infected with *S. aureus* and also a strain of *E. coli.* At the median protective dose (PD_{50}) for these infections the peak serum concentrations of benzyl penicillin, penicillin V, ampicillin and amoxycillin were substantially lower than the respective MICs and a marked inhibitory effect of sub-MIC concentrations could also be demonstrated *in vitro* over the first 6 h of incubation. The reason for these effects may lie in the fact that the members of a bacterial population are not necessarily uniform in their level of antibiotic susceptibility. With a conventional MIC determination, after overnight incubation, the result reflects the susceptibility of the most resistant members but a large proportion of the population may well be inhibited by antibiotic concentrations significantly below the MIC and in many experimental infections in animals merely a reduction in the number of bacteria present may be sufficient to allow the body to overcome the infection. However, although sub-MIC concentrations of antibiotics can exert a significant antibacterial effect *in vitro* and *in vivo* it may well be that in the treatment of serious infections in humans it would be desirable that serum concentrations should exceed the MIC, although the exact relationship between serum concentrations and therapeutic effect is not well established.

β-Lactamase induction and the derepressed mutants, 1980–1989

P. aeruginosa produces a β-lactamase classified by Richmond & Sykes[72] as type Id. In naturally occurring isolates, this β-lactamase was considered typically inducible, but in 1980 my colleague M. N. Gwynn and I[73] reported that cultures of *P. aeruginosa* contained a minority population of variants (derepressed mutants) which produced this enzyme constitutively. As a result of the enhanced level of β-lactamase production these variants showed a high level of resistance to certain β-lactam antibiotics including azlocillin and piperacillin.

In a further report we[74] showed that variants constitutively producing high levels of type Id β-lactamase could also be isolated from cultures of *Enterobacter cloacae* and *Citrobacter freundii.* These variants showed resistance not only to penicillins such as azlocillin and piperacillin but also to third-generation cephalosporins and to latamoxef (Table II). Similar findings were reported by other workers.[75,76]

Emergence of variants of *P. aeruginosa* and *Enterobacter* spp. showing enhanced production of β-lactamase constitutively was also encountered clinically. Following therapy with various β-lactam antibiotics a number of authors reported the isolation of such derepressed mutants with evidence of resistance to one or more β-lactam antibiotics including azlocillin, mezlocillin, piperacillin, cefamandole, cefotaxime, ceftriaxone and cefoperazone.[77–82]

The emergence of stable, multi-β-lactam-resistant variants of *P. aeruginosa* and *Enterobacter* spp. following therapy with β-lactam antibiotics aroused considerable

Table II. Antibiotic susceptibility of derepressed mutants isolated from strains of *Pseudomonas aeruginosa, Citrobacter freundii* and *Enterobacter cloacae* (data from Gwynn & Rolinson[74])

	MIC (mg/L)					
	carbenicillin	azlocillin	piperacillin	cefotaxime	cefoperazone	latamoxef
P. aeruginosa						
parent strain	50	25	5	10	5	10
mutant	125	>500	>500	>100	>100	>100
C. freundii						
parent strain	2.5	12.5	2.5	0.12	0.25	0.12
mutant	250	>500	250	50	100	12.5
E. cloacae						
parent strain	1.25	25	12.5	0.25	5	0.12
mutant	50	>500	250	100	50	5.0

concern.[83] Unfortunately, misunderstanding also arose at this time concerning β-lactamase induction on the one hand and the mutations responsible for the origin of derepressed variants on the other. In *P. aeruginosa, Entero - bacter* and *Citrobacter* spp., β-lactamase type Id is usually produced inducibly. In the absence of an inducer the level of β-lactamase production is low but in the presence of an inducer the level of production is increased. Many β-lactam antibiotics function as inducers; some are good inducers, others are weak inducers. However, induction is a strictly temporary phenomenon and does not lead to the emergence of stable resistance. When the inducer is removed the rate of β-lactamase production returns to a low level. In contrast, the derepressed mutants are the result of a genetic change in which the induction mechanism has been lost. Such mutants produce a high level of β-lactamase constitutively and the resulting resistance to β-lactam antibiotics is stable.

Despite the fundamental difference between β-lactamase induction on the one hand and constitutive enzyme production on the other, a misconception arose that induction was itself responsible for the origin of derepressed mutants. As a consequence, claims were made in favour of β-lactam antibiotics that are poor inducers on the grounds that they would be less likely to lead to the emergence of stable resistance. This misconception continued for a number of years. Efforts to clarify this subject were made in reports by the author[84,85] and by others, notably David Livermore, in which it was emphasized that stable multiple β-lactam-resistance emerges in the clinic as a result of the selection of pre-existing derepressed mutants present as a minority population in *P. aeruginosa* and *Enterobacter* spp. and not as a result of β-lactamase induction.

β-Lactamase inhibitors, 1967–1994

In the mid-1960s, β-lactamase production had not yet appeared in *Haemophilus influenzae, Moraxella catarrhalis, Enterococcus* (then *Streptococcus*) *faecalis* or the gonococcus, but it was already clear that this enzyme would become the major mechanism of resistance to the aminopenicillins. An increase could already be seen in the frequency of β-lactamase-producing ampicillin-resistant strains of *E. coli* and this enzyme was clearly the mechanism of ampicillin-resistance in a number of other pathogens including staphylococci, *Klebsiella* spp. and *Bacteroides fragilis.*

A possible approach to the problem of β-lactamase-mediated resistance was the use of a β-lactamase inhibitor. In 1967 I began a programme of work in which microorganisms were examined for the possible production of naturally occurring inhibitors of β-lactamase. The microorganisms were grown in liquid culture and samples of the broth then tested for β-lactamase inhibitory activity. The test that was used involved the preparation of agar plates inoculated with a culture of *K. aerogenes*. The agar itself contained a concentration of benzyl penicillin such that the β-lactamase activity of the klebsiella was able to bring about inactivation of the antibiotic during subsequent incubation and allow bacterial growth. Before incubation of the plate, broth samples to be tested for β-lactamase-inhibitory activity were placed in wells cut in the agar. If β-lactamase-inhibitory activity was present, the inactivation of the benzyl penicillin was prevented and a zone of inhibition of bacterial growth could be seen surrounding the well. A photograph of such a plate (Figure 3) appeared in an article which I published later[86] describing the history and background to co-amoxiclav.

Using this plate test, β-lactamase inhibitory activity was first detected in a strain of *Streptomyces olivaceous.* This activity was found to be due to the production of a new family of β-lactam compounds which were named olivanic acids.[87] The olivanic acids did not find a place as β-lactamase inhibitors in clinical practice but further work led to the discovery of clavulanic acid[88] produced by a strain of *Streptomyces clavuligerous.* A formulation of clavulanic acid with amoxycillin (co-amoxiclav) was introduced in 1981 and, shortly afterwards, a formulation of clavulanic acid with ticarcillin was introduced.

Not surprisingly, the discovery of clavulanic acid and its introduction into clinical practice stimulated work in other laboratories which led to further β-lactamase inhibitors including sulbactam and tazobactam. The evolution of β-lactamase inhibitors has been reviewed by the author[89] together with a review of the microbiology of co-amoxiclav over the 15 year period 1978–1993.[90]

Reflections

The β-lactams currently present a highly developed field both in terms of the agents available for clinical use and in the understanding of much of the associated basic science. However, many of today's developments have their roots in studies carried out much earlier. For example, the isolation of 6-APA, which made possible the development of the semisynthetic penicillins, goes back 40 years and indeed the incentive for the work which led to 6-APA can be traced back to the efforts made to exploit the penicillin molecule in the 1940s and 1950s by the chemical modification of penicillin X[91] and the preparation of biosynthetic penicillins using side-chain precursors in the fermentation process.[92] Similarly, the programme of work that led to the discovery of the β-lactamase inhibitor clavulanic acid was begun over 30 years ago and the recognition of the clinical potential for a β-lactamase inhibitor can be traced back to studies carried out as early as the 1940s.[93,94]

With regard to present-day concepts and criteria in the β-lactam field a number of examples can also be cited

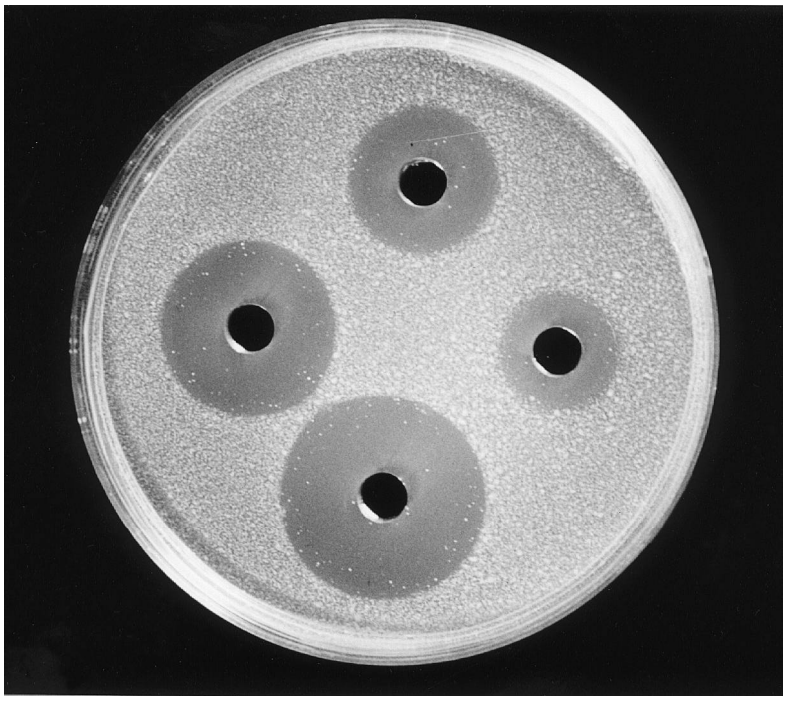

Figure 3. Plate test for β-lactamase-inhibitory activity (agar containing penicillin G and seeded with *K. aerogenes*. Wells contained clavulanic acid 5.0, 2.5, 1.25 and 0.6 mg/L. (Reproduced from Rolinson[86])

where the basis can be found in very early studies. For example, current thinking regarding dosage schedules of β-lactam antibiotics has its origin in the studies, in the late 1940s and early 1950s, by Harry Eagle, who concluded that the therapeutic efficacy of penicillin is determined primarily by the period of time for which the drug remains at an effective concentration at the site of infection.[95,96] Similarly the PAE, about which a good deal is published today, was first described 50 years ago.[58]

It must also be said, of course, that major advances have been made in recent years concerning antibiotic modes of action and resistance mechanisms, notably in the fields of molecular biology and genetics. However, there are also aspects of antibacterial chemotherapy in which our understanding has not advanced very greatly. For example, although the phenomenon of heterogeneity shown by many strains of MRSA was first described over 30 years ago, it is still not clear why resistance to methicillin is expressed in only a small proportion of the population when incubated at 37°C. A further example concerns the growth of bacteria in biofilms. Although the importance of such growth has long been recognized, the precise reason for the diminished antibiotic susceptibility of bacteria under these circumstances remains to be established. There are also a number of broader aspects of antibacterial chemotherapy in which progress over the last 50 years cannot be said to be very marked including the question of optimum dosage and duration of treatment, rational drug usage and antibiotic susceptibility testing.

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