BETA-LACTAM ANTIBIOTICS

Edited by
SUSUMU MITSUHASHI

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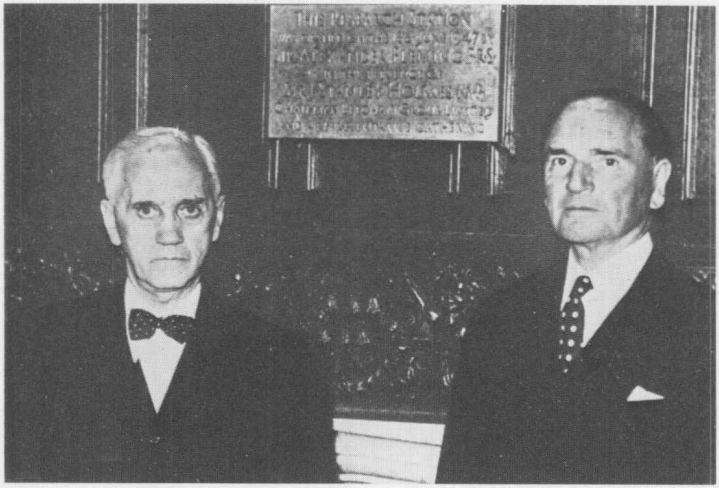
PREFACE

Paul Ehrlich opened the way for the study of experimental chemotherapy and introduced trypanred and salvarsan. His term "Chemotherapy" denotes the treatment of parasitic disease by direct chemical attack upon the invading organisms and the discovery of appropriate chemotherapeutic agents came about through human ingenuity. Therefore, advances in the field of chemotherapy were rather slow. Ehrlich's discoveries were followed by the advent of prontosil (G. Domagk, 1935) and of sulfonamide (G. Tréfouël, 1935), furazolidon (M. Dodd, 1947), *p*-aminobenzoic acid derivatives (PAS, isoniazid), furatrizine (K. Miura, 1961), nalidixic acid (G.Y. Lesher, 1962), *etc.*

The discovery of penicillin by Sir Alexander Fleming in 1929 was accidental but revolutionary. In 1938 Chain, Florey, and their associates purified the active principle from a culture filtrate of *Penicillium notatum*. The introduction of penicillin revolutionized the treatment of many infectious diseases and confirmed that many new chemotherapeutic agents may be found in organisms living in our surroundings, *i.e.*, the soil, water, air, *etc.*

As practical example, Selman A. Waksman isolated streptomycin from *Streptomyces griseus*, a known soil organism, in 1943. In 1947, the antibiotic properties of chloramphenicol were first reported by J. Ehrlich, and Y. Koyama reported colistin in the same year. B.M. Duggar obtained chlortetracycline from *Streptomyces aureofaciens* in 1948. Thereafter, many antibiotics have been introduced in the treatment of infectious diseases, making our era the golden age of chemotherapy in the long history of medicine.

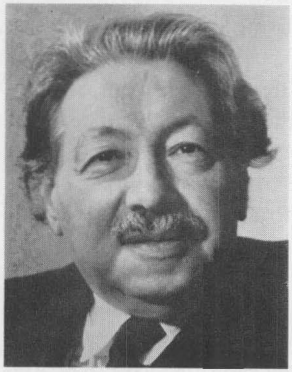
Half a century has passed since the discovery of penicillin and we are now faced with multiply-resistant microorganisms, which are resistant to chemotherapeutic agents formerly referred to as "magic bullets" in medicine and the livestock- and



Sirs Alexander Fleming (left) and Stanley Holmes (right).

fish-breeding industries. The discovery of drug-resistance plasmids in Japan has clarified the reason for the rapid spread of multiply-resistant organisms all over the world, and in addition studies of drug-resistance plasmids have told us that wide gene exchanges occur among microorganisms through plasmids and among replicons through transposons.

Soon after returning from the Second World War, I was asked by Prof. S. Hosoya to begin studies on penicillium culture and penicillin purification during the period of Allied Occupation. We noticed that penicillin's activity was suddenly lost in some cases when culture filtrates of different bottles were combined together. I noticed accidentally that the destruction of penicillin was due to contamination of gram-negative bacteria from potatos used for the preculture of penicillium. Owing to the advice of Prof. F. Egami, I purified the enzyme, penicillinase, without knowing of the published report on penicillinase by E.P. Abraham and E. Chain, because all literatures from foreign countries were only available in the U.S. Army Library in Japan. This was my first finding during the 35-year history of my life in research. Therefore, I have accepted with pleasure the task of editing this monograph on "Beta-Lactam Antibiotics" at the half century mark after the discovery of penicillin. The editor appreciates very much the support of all contributors, who are among the most eminent scholars in the world.



Professor Ernst B. Chain

I had asked Ernst B. Chain to write the foreword for our book. It was indeed unfortunate that he died suddenly last summer soon after he had accepted with pleasure.

April 1981

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Department of Microbiology
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FOREWORD

The body of scientific knowledge, like the murein sacculus, is a seamless web with many levels: each component is connected directly to a few others, and indirectly to all. The most spectacular connection of penicillin, is of course, to therapy, where its dramatic action has surely saved millions of lives. But this molecule is also remarkable for the variety of its other direct connections: *i.e.*, the principles, applications, and novel areas of study that it has generated. In industry penicillin initiated the systematic search for additional antibiotics, the isolation of mutants that increased the yield, and the development of aerobic "fermenters" with unprecedented capacities. Antibiotics have also made possible daring new kinds of major surgery, and they have advanced both virology and the study of cell biology by greatly simplifying animal cell culture. Organic chemists have found a wealth of challenging problems in the β-lactams, with their novel 4-membered ring; and the degradation products of these complex molecules have made possible model studies on the development of allergy to haptens.

In microbiology β-lactams continue to generate exciting new leads. Penicillin demonstrated earlier the remarkably selective toxicity of an inhibitor of a reaction unique to the parasite; it introduced the use of antibiotic resistance as a selective genetic marker; and its interference with a specific reaction in peptidoglycan synthesis was indispensable for the isolation of pieces small enough to analyze. More recently, refined studies have shown that β-lactams inhibit a number of different transpeptidases in the cell, and the regulation of these enzymes may well provide the key to the morphogenetic processes that divide a bacterial cell and determine its shape.

Perhaps the broadest contribution of antibiotics to biology has been the recognition that plasmids are ubiquitous, as originally shown in Japan. Moreover, they code not only for drug resistance but also for many other functions that are optional for the cell. Their use in molecular recombination of DNA *in vitro*, and in the study of special insertion sequences, is revolutionizing molecular genetics. Their role in

gene transfer in evolution is increasingly recognized, and it may not be restricted to bacteria. Finally, studies with a variety of β-lactams are revealing a novel property of some enzymes, in which exposure to one ligand induces a relatively persistent conformational change that alters the subsequent reaction with another.

It is thus clear that work on β-lactams has led not only to immediate practical applications but also to many unexpected spinoffs. These provide strong support for two principles that need constant reiteration: basic and applied research are inextricably intertwined; and the most interesting and valuable discoveries are often unpredictable. This was true of Fleming's original observation; it was true of Chain's decision to purify a lytic agent that he assumed was an enzyme; and it will no doubt be true of many further discoveries involving the β-lactams.

Bernard D. Davis
Bacterial Physiology Unit
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1 HISTORY

A SHORT HISTORY OF THE β-LACTAM ANTIBIOTICS

E. P. ABRAHAM

*Sir William Dunn School of Pathology**

THE DISCOVERY OF PENICILLIN AND ITS CHEMOTHERAPEUTIC PROPERTIES

It is now more than half a century since Alexander Fleming observed the antibacterial action of penicillin on a plate seeded with staphylococci at St. Mary's Hospital, London, and nearly forty years since the work of Florey and Chain and their colleagues in Oxford demonstrated the remarkable therapeutic power of penicillin that led to its widespread use in medicine. These discoveries proved to be the forerunners of others which have led to the production of a large family of antibiotics, with established or potential clinical value, whose single common structural feature is the possession of a β-lactam ring. In this introductory chapter an attempt will be made to survey briefly how such developments came about.

Several aspects of the early work are worthy of comment, for numerous popular accounts of what happened have been notable mainly for the extent of their divergence from reality. Fleming's original observation of the production of penicillin after an accidental contamination of his plate on a laboratory bench by *Penicillium notatum* is not easily repeatable. Penicillin brings about the lysis of growing staphylococci, but not of resting organisms, and it appears that only unpredictable changes in the summer temperature made it possible for the fungus to grow and produce penicillin before the bacteria had completed their growth and become insensitive. However, Fleming cultured the fungus in broth and showed that the culture filtrates, which he called penicillin, were highly active against a variety of gram-positive bacteria. He also showed that the active broth was no more toxic than ordinary broth to animals and to leucocytes and used it in a few cases as a local antiseptic; but his interest in it for this purpose clearly waned, for he wrote in 1940: "the trouble of

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making it seemed not worth while." The idea that it might be introduced into the bloodstream and function as a systemic chemotherapeutic agent apparently never occurred to him.

Attempts to isolate penicillin in the decade following its discovery were soon abandoned, mainly because of difficulties arising from its instability. But, in 1938, the question was reopened when H. W. Florey and E. B. Chain decided to make a systematic investigation of antimicrobial substances produced by microorganisms in the Sir William Dunn School of Pathology, Oxford. This decision was a consequence of their interest in lysozyme. Penicillin was fortunately one of the first substances chosen for study, and the work of a small group of people led to its purification and to the demonstration by Florey and his colleagues of its remarkable therapeutic properties, first in mice and then in humans.

Despite the gratifying outcome of this work, which seemed at the time to be almost miraculous, the production of penicillin in war-time Britain in the quantities needed for its general medical use posed a virtually insoluble problem. Florey therefore went to the United States with N. G. Heatley, in 1941, to enlist American help. Subsequent work in the Northern Regional Research Laboratories in Peoria and in several American pharmaceutical companies was responsible for the addition of corn-steep liguor to the growth medium, the introduction of deep fermentation, and the isolation of higher-yielding strains of *Penicillium* which entirely changed the outlook for the large-scale production of penicillin. Thus, enough penicillin became available to treat all serious British and American casualties during the invasion of Europe in 1944.

During the war years the chemistry of penicillin became the subject of a massive and confidential Anglo-American investigation, involving many commercial and academic organisations, whose ultimate aim was to produce penicillin in quantity by chemical synthesis. The sodium salt of benzylpenicilin was crystallised at the Squibb Institute for Medical Research by Wintersterner and MacPhillamy in 1943 and soon afterwards a crystalline sodium salt was obtained from the purified 2-pentenylpenicillin of Abraham and Chain in Oxford. The β-lactam structure for penicillin (Fig. 1) was first proposed by Abraham and Chain in 1943. It was later strongly supported by R. B. Woodward, but was opposed by those committed to an alternative thiazolidine-oxazolone structure. However, the β-lactam structure was finally established beyond doubt in 1945 when an X-ray crystallographic analysis by Dorothy Hodgkin and Barbara Low came to a successful conclusion.

The outcome of the synthetic work during this time was disappointing, for no more than trace amounts of penicillin were ever obtained, and these in attempts to synthesise the thiazolidine-oxazolone structure.

By 1948 the outstanding value in medicine of benzylpenicillin had been firmly established. Many of its limitations, including a relatively low activity against gram-negative bacilli and the tubercle bacillus, were also known. In 1940 the first penicillinase had been discovered in *Escherichia coli* by Abraham and Chain, who suggested that the production of this enzyme was responsible, in some cases though not in

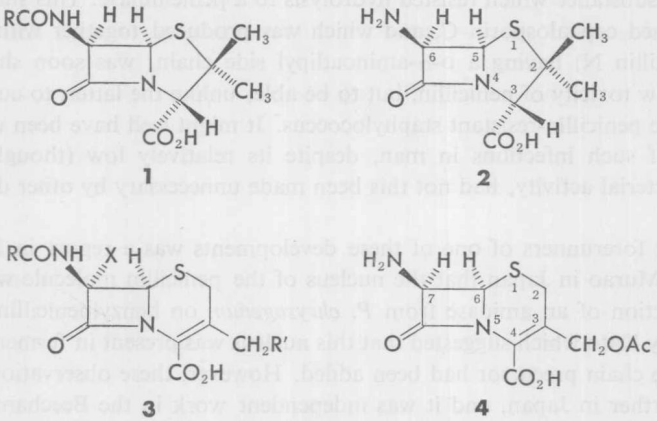

FIG. 1. Structures of: 1, penicillins; 2, 6-APA; 3, cephalosporins; and 4, 7-ACA. R is variable in the penicillins. In the cephalosporins R and R′ are variable and X is H or OMe.

others, for bacterial resistance to penicillin. Within five years penicillinase had been found to be produced by a number of gram-negative and gram-positive pathogens, including some strains of *Staphylococcus aureus*, and in one case had been shown by Duthie to be an inducible enzyme. By this time it had also been shown that penicillins with different nonpolar side chains derived from mono-substituted acetic acids could be obtained by addition of appropriate side chain precursors to the fermentation of *Penicillium chrysogenum*; but none of the penicillins thus obtained had proved to be markedly superior to the benzylpenicillin then in use, although in 1954 the discovery of the relative acid-stability of phenoxymethylpenicillin (penicillin V) made the latter the first penicillin suitable for oral administration. Thus, towards the end of the 1940's the history of penicillin might have been regarded as that of a great medical discovery to which little more would be likely to be added. In the event, there were to be remarkable and unforeseeable further developments. These were to lead to the production of thousands of new β-lactam compounds, a number of which were to be responsible for significant advances in chemotherapy.

CEPHALOSPORIN C, 6-AMINOPENICILLANIC ACID, AND 7-AMINOCEPHALOSPORANIC ACID

In the early 1950's a serious clinical problem arose from the emergence of penicillinase-producing staphylococci and the failure of the penicillins then available to cope with infections caused by these organisms. It was thus of some interest when Newton and Abraham, who were studying the production of antibiotics by a species of *Cephalosporium* that had been isolated by Brotzu in Sardinia, discovered in 1953

a penicillin-like substance which resisted hydrolysis to a penicillinase. This substance, which they named cephalosporin C, and which was produced together with a new penicillin (penicillin N) having a D-α-aminoadipyl side chain, was soon shown to have the very low toxicity of penicillin, but to be able, unlike the latter, to cure mice infected with the penicillin-resistant staphylococcus. It might well have been used for the treatment of such infections in man, despite its relatively low (though wide-ranging) antibacterial activity, had not this been made unnecessary by other developments.

The earliest forerunners of one of these developments was a report in 1950 by Sakaguchi and Murao in Japan that the nucleus of the penicillin molecule was produced by the action of an amidase from *P. chrysogenum* on benzylpenicillin and a report in 1953 by Kato which suggested that this nucleus was present in fermentations to which no side chain precursor had been added. However, these observations were not explored further in Japan, and it was independent work in the Beecham Laboratories by Rolinson and others which resulted, by 1959, in the isolation of the penicillin nucleus (6-aminopenicillanic acid, 6-APA, in Fig. 1) in quantity.

At about this time a definitive structure for cephalosporin C [Fig. 1, R=$^{-}O_2CCH(N^{+}H_3)(CH_2)_3$, R′=$OCOCH_3$] was proposed by Abraham and Newton and confirmed, soon afterwards, by an X-ray crystallographic analysis by Hodgkin and Maslen. It was also found that the nucleus of cephalosporin C (7-aminocephalosporanic acid, 7-ACA, in Fig. 1) could be obtained, although in very small yield, by acid hydrolysis under controlled conditions and that acylation of 7-ACA with phenylacetyl chloride yielded a cephalosporin with very much higher activity than cephalosporin C itself against gram-positive bacteria. Furthermore, it was shown that the acetoxy group at C-3′ in cephalosporin C could readily be replaced by a variety of nucleophiles to give compounds with higher activity. These compounds retained a resistance to hydrolysis by staphylococcal penicillinase, the resistance being due to the nature of the ring system.

SEMI-SYNTHETIC NEW PENICILLINS AND CEPHALOSPORINS

The finding that penicillin N, unlike benzylpenicillin, was as active against a number of gram-negative bacteria as it was against gram-positive organisms indicated that penicillins with other side chains, differing from those of the penicillins obtainable by fermentation of *P. chrysogenum*, might have interesting biological properties. Soon after the isolation of 6-APA, the Beecham Group began a fruitful study of new penicillins that could be prepared from it by chemical acylation. This led first to methicillin (R=2:6-dimethoxyphenyl in Fig. 1), which largely solved the clinical problem of the penicillin-resistant staphylococcus because of its stability in the presence of staphylococcal penicillinase. A major factor here was the very poor affinity of methicillin for the staphylococcal β-lactamase, which was associated with the presence of the two bulky methoxyl groups in its side chain close to the β-lactam ring. Methicillin was followed by oxacillin and cloxacillin which also resisted hydrolysis

by the staphylococcal enzyme but which, unlike methicillin, were relatively stable to gastric acidity and were absorbed when given by mouth.

The first new penicillins showed very low activity against gram-negative bacteria. However, in 1961 ampicillin, with an amino group attached to the α-carbon atom of its side chain, was found to be considerably more active than benzylpenicillin against many gram-negative organisms. This α-aminobenzylpenicillin has been succeeded by the closely related amoxycillin. Both compounds are effective in the treatment of infections by a variety of gram-negative bacteria, but not infections by the penicillin-resistant staphylococcus, since they are readily hydrolysed by staphylococcal penicillinase.

While valuable new semi-synthetic penicillins were being produced by the Beecham Group the discovery in the Lilly Research Laboratories in 1962 of a chemical method for the removal of the α-aminoadipyl side chain of cephalosporin C in good yield and the production of 7-ACA in quantity opened the way to the extensive exploration by pharmaceutical companies of the potentialities of the cephalosporin ring system. A year later the Lilly group described a chemical route from the penicillin to the cephalosporin ring system which also proved to be of major importance.

Cephalothin, with a thienylacetyl side chain, was the first semi-synthetic cephalosporin to come into medical use. It was effective against a variety of gram-positive bacteria, including penicillinase-producing staphylococci, and also against a number of gram-negative bacilli. Cephalothin was followed by Glaxo's cephaloridine, in which the acetoxy group at C-3′ of cephalothin was replaced by a pyridinium group. The latter was not removed by acetyl esterases which converted cephalothin to the less active deacetylcephalothin *in vivo*. Later cefazolin was produced by Fujisawa in Japan and was the first of a number of cephalosporins in which the acetoxy group was replaced by a heterocycle carrying a thiol group.

The early cephalosporins were not absorbed from the gastrointestinal tract, despite their relative stability to acid. However, cephalosporins with a D-phenylglycyl side chain (that of ampicillin) were readily absorbed when given by mouth. The first of these compounds, introduced by Eli Lilly, was cephalexin, which also contained a methyl group in place of an acetoxymethyl group at C-3′. A related compound, cephradine, containing a cyclohexadienylglycyl side chain, was then produced by Squibb.

The availability and use of β-lactam antibiotics able to cope with infections by gram-positive bacteria, together with changing patterns of infection, focused attention on a growing problem of resistance in gram-negative bacteria. In some cases and particularly with *Pseudomonas aeruginosa*, resistance was partly due to the poor ability of β-lactam antibiotics to penetrate the cell wall and reach their sites of action on the cytoplasmic membrane. But another important factor was the ability of many of the resistant organisms to produce a cell-bound β-lactamase. It became evident that there were many β-lactamases with different substrate profiles and that while some of them were chromosomal others were mediated by plasmids which could be transferred from one organism to another.