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REVIEW

Different roads to discovery; Prontosil (hence sulfa drugs) and penicillin (hence β-lactams)

Ronald Bentley

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It is a rough road that leads to the heights of greatness (Seneca).

Abstract The important chemotherapeutic agents, Prontosil and pentenylpenicillin (penicillin F), were investigated initially by two men, Domagk and Fleming, who had been influenced by the horrendous wound infections of World War I. The very different pathways leading to their development and to that of the successor antibacterials (sulfa drugs, further penicillins, semi-synthetic penicillins), including the role played by patents, are discussed.

Keywords Prontosil · Penicillins · Sulfanilamide · Sulfa drugs · Semi-synthetic penicillins · Submerged fermentation · Patents · Domagk · Fleming · Florey · Chain

Two important medicinal developments of the past eight decades were the discovery and utilization of the antibiotics, Prontosil (leading to the sulfa drugs) and penicillin (leading to related antibiotics). Although Prontosil is not a microbial product, it may be regarded as an antibiotic under the "big tent definition" [7]. The significance of these developments is confirmed by the award to their investigators of a total of four Nobel Prizes for Physiology or Medicine. Together, Prontosil, penicillin, and related antibacterials enabled physicians to control the major infectious diseases afflicting humanity, at least for many decades; however, an increasing problem today is the steady development by bacteria of resistance to antibiotics. It is instructive to consider the very different processes by which these two antibiotics, and the succeeding sulfa drugs and β-lactams, were brought into use.

R. Bentley
Department of Biological Sciences,
University of Pittsburgh, Pittsburgh, PA 15260, USA

R. Bentley (✉)
37 Thornwood Drive, Pittsburgh, PA 15228-2452, USA
e-mail: rbentley@pitt.edu

The beginnings

It is reasonable to claim that in both cases these two antibiotics had a distant but common genesis; their developments were rooted in the horrendous battle injuries suffered by the armies of World War I. These injuries were frequently magnified by the subsequent bacterial infection of the wounds. With primitive field hospitals, the work of dedicated and skillful surgeons was often futile because, despite the liberal use of antiseptics such as cresol, their efforts were defeated by the subsequent infections. Thus, in October 1914, the Director General of the British Army Medical Service stated "We have in this war gone straight back to all the septic infections of the Middle Ages" [11]. Poorly nourished civilian populations were also highly susceptible to bacterial infections, and in those days childbed fever (puerperal fever) was a constant threat to women who gave birth.

Their experiences during World War I made a profound impression on many physicians and others in the medical services. Among them was Gerhard Johannes Paul Domagk (1895–1964), who served in the German Army and was himself wounded. He graduated from medical school in 1921. On the Allied side, the Scot, Alexander Fleming (1881–1955) who had graduated from medical school in

1908, served in the Royal Army Medical Corps. These two individuals, using totally different methods, provided the first effective antibacterial materials. There is detailed biographical material on Domagk [12, 18] and on Fleming [11, 38].

Prontosil: early work to 1929

In the late 1920s, the Friedrich Bayer Company, a member of the IG Farben cartel, began extensive research on the possibility of treating bacterial diseases with synthetic chemicals. This pharmaceutical research program, headed by Heinrich Hörlein, was inspired by Ehrlich's methods and success in using arsenical compounds such as Salvarsan and Neosalvarsan in the treatment of syphilis. In 1927, Domagk was recruited into Bayer's carefully organized system for the production of new drugs. As a result of his war-time service, he had witnessed first-hand the struggle against wound infections. He joined a massive, well-designed, methodical team effort, supported by the deep pockets of a prosperous company already making many chemical products for the medical market (e.g., Aspirin, Luminal) and the dyestuffs industry. The ultimate aim was patentable and marketable pharmaceutical materials.

Potential antibacterials were synthesized by chemists and tested by Domagk both on animals and in vitro. In particular, as a test organism he used a highly virulent Streptococcal strain, *Streptococcus hemolyticus*, isolated from a human case of "streptococcus sepsis". Many assistants and animal-care support staff were involved. By 1929, thirty new products could be tested per week [30]. A similar group, often using the same materials, worked on tropical diseases.

Penicillin: early work to 1932

The complex, even bizarre, character, Almroth Wright (1861–1947), was appointed Professor of Bacteriology at St Mary's Hospital in 1902. His dominant personality has been vividly described by Dunnill [25]. He had developed an anti-typhoid vaccine that saved the lives of many soldiers. In 1914 as World War I began, he was appointed as a Lieutenant-Colonel, and established a laboratory for the study of wound infections in Boulogne. His team included Lieutenant Alexander Fleming. In France, he and Fleming rejected the use of the Listerian tradition of using strong antiseptics (such as cresol) to treat war wounds. They promoted the use of a drainage tube and hypertonic saline—a technique widely used again in World War II.

One of Fleming's earlier experiences was in 1909 when Ehrlich had given Wright a sample of Salvarsan. Wright, a proponent of vaccines, asked Fleming and another colleague, Leonard Colebrook, to work with it. For a time, Colebrook and Fleming were the only physicians in England using this chemotherapy. Following his war-time service, Fleming returned to the Inoculation Department of St Mary's Hospital in 1919, with the reputation of an expert in the bacteriology of wound infections. The Inoculation Department was an unusual unit, a semi-autonomous research institute (with its own patient ward) within a general hospital. A major level of financial support came from the sale of a variety of vaccines, with further support from wealthy friends of the Director, Almroth Wright, and from private patients. The facilities, which have been described in detail [11, 31] were far from ideal. In 1921, Fleming became Assistant Director of the Inoculation Department and Director of the Department of Systematic Bacteriology in the Pathological Institute of St Mary's Hospital. While Wright was unsympathetic to any possibility of chemotherapy, Fleming, with his Salvarsan experience was more receptive.

In 1921, Fleming serendipitously discovered in his own nasal mucosa, a material that "dissolved" certain bacteria, especially one named first as *Micrococcus lyticus* and later as *Micrococcus lysodeikticus.* However, the virulent forms of staphylococci, streptococci, etc., were not affected by this material and there were no indications that it had any application in the treatment of infections. However, this material, named lysozyme, later played a role in the penicillin story.

In 1928, while studying staphylococcal variants for a monograph to be produced by the Medical Research Council (MRC), Fleming found a Petri dish of staphylococci on which a contaminant fungus was present. In the vicinity of the fungus, there was extensive bacterial lysis. This second, serendipitous discovery was the beginning of the penicillin saga. Fleming named his "mould broth filtrate" [27] as penicillin, deriving this trivial name from the name of the fungus, then believed to be *Penicillium rubrum*. He used the filtrate from growth of the fungus to treat a number of infections, but gave few experimental details. Similar crude preparations were used to treat eye infections by C.G. Paine at The Royal Infirmary, Sheffield; clinical records of this work have survived [56]. While Fleming appreciated that there were possible uses for penicillin as an antibacterial agent, he made no significant further progress. Fortunately, he did maintain the fungus in culture.

In 1932, Harold Raistrick and his colleagues at the London School of Hygiene and Tropical Medicine, reinvestigated the metabolic products of *Penicillium chrysogenum*, including "penicillin, the antibacterial substance of Fleming" [17]. Raistrick, a distinguished natural products chemist, identified Fleming's fungus as a strain of

Penicillium notatum, showed that it could be grown on a chemically defined medium rather than on Fleming's "broth", and determined that penicillin was an acid that could be extracted into ether. However, when ether solutions were evaporated to obtain the active material, a standard isolation process in working with natural products, the antibacterial activity was lost. Daunted by this chemical instability, Raistrick did not pursue his work; moreover, his medical colleagues advised him that an unstable material "would never be of practical use in clinical medicine" [10]. For several years, penicillin retreated to the depths of library shelves.

Prontosil: 1929 forward

Progress in obtaining an antibacterial agent at Bayer was painfully slow. After abandoning work with quinine derivatives and compounds containing gold, the chemist, Josef Klarer, began work with azo dyes. Such dyes were well known to bind to various cells and some were used as bacterial stains. The results were uneven, sometimes not reproducible. At some time in 1932, Hörlein, apparently based on his earlier work with azo dyes, suggested that addition of sulfur atoms might be worthwhile. It was a most productive suggestion. Klarer delivered a sulfur-containing azo dye to Domagk in early October, 1932 [30]. Its code number in Klarer's laboratory was Kl-695 (Fig. 1a). While this material was without activity against streptococci in vitro, surprisingly it protected infected mice. Domagk stated in 1936 that one of the first materials with a good chemotherapeutic action against streptococci in mice was Kl-695 [23]. Further synthetic work yielded, Kl-730 (Fig. 1b), as an even more effective and consistent antibacterial against Streptococci in mice. Kl-730 is the hydrochloride of 4-[(2,4)-diaminophenyl)azo]benzenesulfonamide, also known as sulfamidochrysoidine. This compound was synthesized by diazotization of 4-aminobenzenesulfonamide (sulfanilamide) followed by coupling with *m*-phenylenediamine. The preparation was described in a German patent application by F. Mietzsch and J. Klarer (I.G. Farbenindustrie) submitted December 25, 1932 and granted December 13, 1934 [39].

Extensive testing showed that this red dye was a very potent material in treating streptococcal infections in animals, although it was inactive in vitro. Domagk described the work in a 1935 paper that is now a classic [22]. However, it is not clear how this dye, Kl-730, came to be named Prontosil. In his 1935 paper, Domagk states that among the non-toxic, azo antibacterials "was Prontosil, which Mietzsch and Klarer had synthesized in 1932". He noted further that "Prontosil will be tested in the clinic under the name 'Streptozon'". This seems to imply that the name was

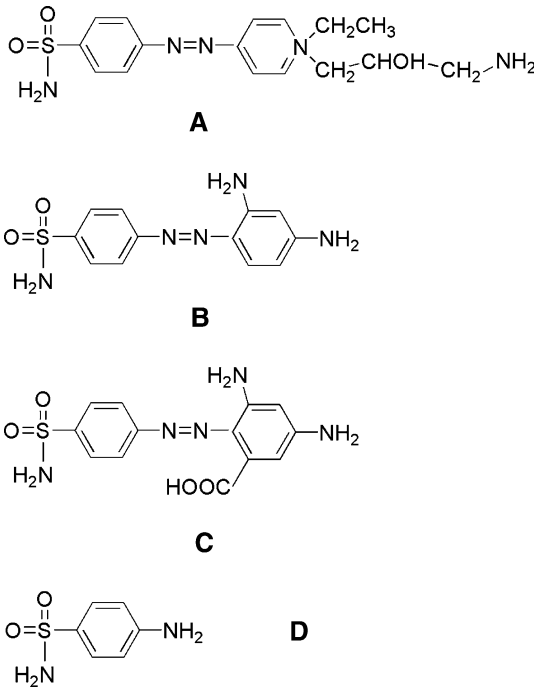

Fig. 1 Prontosil and related compounds. **a** compound Kl 695, **b** compound Kl 730 (sulfamidochrysoidine); as hydrochloride = Prontosil, **c** Rubiazol (sulfa-chrysoidine), **d** sulfanilamide

in use by 1932. However, individuals such as T. Hager [30] and J. Lesch [35], who have studied materials in the Bayer archives, record that the name Streptozon in place of Kl-730 was first used in Domagk's notebooks on December 20, 1932, and that this name was used in Bayer internal memos through most of 1934. Hager believes that the name was changed to Prontosil as Bayer prepared to market the compound (personal communication). A further complexity was that Kl-730 was also known under a Domagk number as D 4145. The Corporate Historian at the Bayer Archives has kindly informed me that he found nothing in their files to provide the etymology for the name, Prontosil, and for the Streptozon to Prontosil change.

The answers to these questions are presumably hidden in Administrative and Marketing files at Bayer that are not presently available for public viewing [30]. The Bayer Company has a long history of providing trade names for its pharmaceuticals, beginning with Aspirin in 1899. In the latter case, the etymology is known: *a* from *a*cetylierte, *spir* from *spir*säure, with the addition of *in* (spirsäure = salicylic acid).

Thus, by 1936, Domagk and his colleagues had begun to use Prontosil, and a more soluble form Prontosil S, in treatment of human streptococcal infections. A notable case was that of Domagk's own daughter [23]. At this time, Prontosil was slowly being made available internationally. In England, Leonard Colebrook, who had worked with Fleming on the use of Salvarsan (see earlier), had become

interested in the potential of Prontosil and began trials with it in July, 1935. His early experiments with mice were not encouraging although it did have activity against highly virulent streptococcal strains, but not those from human infections [35]. There were also conflicting reports from other investigators in the United Kingdom. The rather confused situation in the United Kingdom, and the role of the Medical Research Council's Therapeutic Trials Committee, has been detailed by Lesch [35]. Eventually, Colebrook, in collaboration with Meave Kenny used Prontosil and Prontosil Soluble to treat human puerperal infections with generally positive results [19].

Sulfanilamide: 1935

In France, the Roussel Laboratories had seen an application for a French patent for the preparation of Prontosil in 1935; they prepared a similar compound named Rubiazol, also known as sulfachrysoidine (Fig. 1c). Also in France, Ernest Fourneau (1872–1949) was principal of a Laboratory of Therapeutic Chemistry at the Institut Pasteur. Fourneau had an impressive chemical background, having worked with Emil Fischer and also with Richard Willstätter in the laboratory of Adolf von Baeyer. He had developed a strong interest in chemotherapy and had published substantial work in the 1920s on arsenicals, some in collaboration with Jacques and Thérèse Tréfouël [32]. By the middle of 1935, Fourneau and his colleagues (the Tréfouëls, D. Bovet, F. Nitti) had duplicated Domagk's work with Prontosil [35]. Moreover, the Tréfouëls prepared new azo dyes, some of which were sulfonamide derivatives. In November of that year, Fourneau's group made the important discovery that sulfanilamide itself (Fig. 1d), code numbered 1162F, was an effective antibacterial agent. The discovery is said to have been serendipitous—for one set of experiments a group of four infected mice was in excess and Bovet suggested that sulfanilamide, the common portion to other molecules being tested, should be tried. However, Lesch has pointed out a discrepancy [35]. In the published report of the work it was noted that they had deduced that the breaking of the azo bond, with formation of sulfanilamide, might be responsible for the antibacterial activity [55]. Sulfanilamide slowly began to be marketed in France.

While this impressive discovery was not made in an industrial setting, Fourneau had extensive connections with industry, including a long-standing association with Établissements Poulenc Frères (later to become a parent company of Rhône-Poulenc and even later to be incorporated into Sanofi–Aventis). He had also been successful in the copying of materials covered by German patents, a legal operation under French law at that time.

Moreover, G.A.H. Butte, W.H. Gray and Dora Stephenson, working at Wellcome Physiological Research Laboratories and Wellcome Chemical Laboratories, reported that they had confirmed and extended the French work with sulfanilamide. By early 1937, sulfanilamide was on the market in Britain, France, and the United States. As Lesch has noted, "One of the remarkable things about this story is that the skepticism about bacterial chemotherapy, so pervasive in early 1935, was everywhere dissipated by the Spring of 1937" [35].

Sulfa drugs: 1935–1945

With the discovery of the antibacterial activity of sulfanilamide, developments in chemotherapy from 1935 to about 1945, remained in the hands of organic chemists, because the readily available sulfanilamide could be easily modified. The first major success for the second generation of sulfa drugs was sulfapyridine (Fig. 2a) prepared by the May and Baker Company as M & B 693 (originally T693 in their test book). This antibiotic, which was very useful in treating pneumonia, was made available generally in 1938 under the trade name, Dagenan (the Company's factory was located in Dagenham). The discovery process and the many inaccurate accounts in the press have been reviewed [35]. One of the M & B drugs, either 693 or the later M & B 760, sulfathiazole (Fig. 2b) was famously used to treat Winston Churchill for pneumonia in 1943. Churchill joked that M & B also referred to his physicians, Lord Moran and Brigadier Bradford [35]. By the end of 1939, sulfapyridine was extensively used to treat pneumonia in many countries.

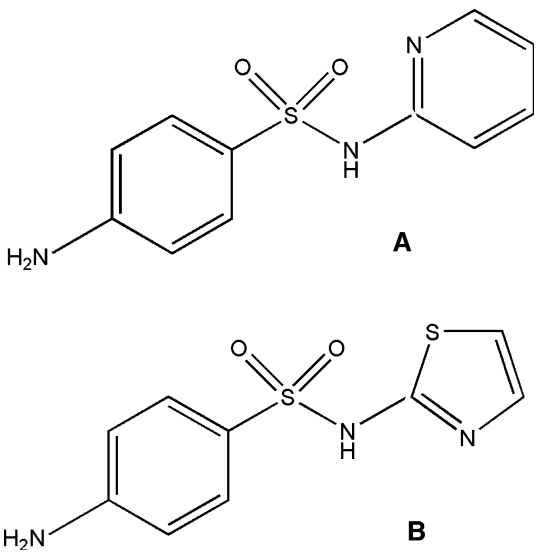

Fig. 2 Sulfa drugs. **a** sulfapyridine (M & B 693), **b** sulfathiazole (M & B 760)

Sulfapyridine was prepared by reacting acetylsulfanilyl chloride with aminopyridine to yield an acetyl derivative. The acetyl group was removed with alcoholic hydrochloric acid. It was soon followed at May and Baker by sulfathiazole. The scope of organic chemistry is impressively illustrated by the fact that more than 5,000 new sulfa drugs had been prepared by 1945 [35]. Domagk has reviewed twenty-five years of sulfonamide therapy [24].

Penicillin: 1935 forward

On May 6, 1935, the British Commonwealth of Nations celebrated the Silver Jubilee of His Majesty George V, by the grace of God, of Great Britain and Ireland, and the British Dominions beyond the Seas, King, Defender of the Faith, Emperor of India. Although that year perhaps represented the high point of the British Commonwealth, it was in another sense the beginning of the end of the Empire. George V died in the following year and by then Adolf Hitler had restored German military might. With the German invasion of Poland in September 1939, World War II began with the eventual breakup of the British Empire.

In that Jubilee year of 1935, the Australian-born physiologist Howard Florey (1898–1968) was appointed to the Chair of Pathology at the Sir William Dunn School of Pathology, Oxford. He had received his medical qualification at Adelaide in 1921. Later, he was to become first Sir Howard and then Lord Florey [9, 36, 59]. An interesting connection with Prontosil is that Florey's daughter, Paquita, developed a serious mastoid infection in 1936. In addition to surgery, she was treated successfully with sulfanilamide [36]. In searching for a biochemist for his biologically oriented research group, Florey received a recommendation from Gowland Hopkins for the German-born, Ernst Boris Chain (1906–1979). Chain, later to become Sir Ernst, was appointed by Florey as Demonstrator in 1935. Florey continued to develop his research group during 1936. His research interests were varied. He had earlier become interested in Fleming's lysozyme and he accordingly encouraged Chain to make a detailed investigation of this material. Chain was able to show that it was an enzyme acting on peptido-glycan heteropolymers of prokaryotic cell walls. Lysozyme has weak antibacterial activity.

By 1938 it was clear from the Prontosil/sulfonamide work that some bacterial infections could be treated by chemotherapy. At this time, Chain and Florey had developed a solid working relationship and to some extent had a common interest in the action of antibacterial substances (unhappily, this relationship deteriorated at a later date). The events leading Florey to focus on penicillin are not entirely clear although their interest in substances such as lysozyme led Chain to carry out a literature search that revealed many examples of microbial antagonisms; these antagonisms are now described as antibiosis. One example that caught Chain's attention was Fleming's penicillin; he believed that, like lysozyme, it might be an enzyme. It is known that Florey had decided to drop work on lysozyme by the end of 1938 and to focus on penicillin [9]. The main focus was initially on the academic problem of antagonism, although Chain and Florey also had possible therapeutic action in mind.

Beginning in 1939, and eventually working under very difficult war-time conditions, Florey and his colleagues constructed a makeshift pilot plant producing salts of impure penicillin in a solid and stable form. All of the necessary fungal growth with Fleming's strain of *Penicillium notatum* was carried out as surface culture in specially designed ceramic containers. Much credit for this achievement is due to the remarkable talents of N.G. Heatley that were put to use in designing the pilot plant and in devising a method for routine bioassay. The clinical potential of penicillin was demonstrated and landmark papers were published in 1940 and 1941 [1, 14]. Because of the difficulties in obtaining materials and supplies under the conditions then facing England (the possibility of a German invasion was a real threat) Florey and Heatley in mid 1941 traveled to the United States and Canada where they catalyzed the massive scale up of penicillin production using improved fungal strains, submerged fermentation conditions, and the use of precursor molecules. This work marked the beginning of the impressive development of the fermentation industries in the production of commodity chemicals [8].

Synthetic penicillin: 1943–1946

Less well known is another aspect of the penicillin saga. Apart from vaccines, the pharmaceutical industry in the early 1940s was essentially based on organic chemistry. The results of the organic chemists in synthesizing the many sulfa drugs, as previously described, were impressive. In this climate, it was inevitable that organic chemists would turn their sights on penicillin. The proposition was simple—if the chemical structure of penicillin was known, organic chemists could doubtless devise a chemical synthesis and remove the need to depend on a pesky, unreliable, and unpredictable fungus requiring complex machinery.

In England, attempts to determine the chemical structure of penicillin began initially in Robert Robinson's Department of Organic Chemistry at Oxford and Ian Heilbron's similar department at Imperial College in London (Imperial College is now named as Imperial College of Science,

Technology and Medicine). In the United States, similar chemical work was largely concentrated in several industrial laboratories. An elaborate administrative structure involving the Office of Scientific Research and Development in Washington, DC, and the Medical Research Council in London, was established in 1943 to manage a collaborative investigation in both the UK and the USA [16, 54]. Because penicillin had potential military value (wounded soldiers who did not develop crippling bacterial infections could be more quickly returned to active duty) publication of information was restricted. This remarkable collaboration eventually involved 39 laboratories, both academic and industrial; a complete list is available [16]. The involvement of the industrial groups required attention to complicated anti-trust and other legal matters so that information could be freely shared to the common benefit. Under this arrangement, approximately 800 reports, some brief and some very long, were produced and circulated in both countries.

By the end of 1945, several major objectives had been achieved. It had been shown that rather than a single penicillin, there was a family of structures having the common antibiotic activity. Pure crystalline samples of penicillins had been obtained and it was shown that in addition to the usual C, H, and O atoms, penicillins contained both N and S (the only chemical feature shared with the sulfa drugs). Moreover, the unusual β-lactam structure had been determined, not without a struggle [6]. However, the objective of a rational and functional chemical synthesis eluded the investigators. As the Nobel Laureate, R.B. Woodward (who had been one of the investigators) noted: "…despite the best efforts of probably the largest number of chemists ever concentrated upon a single objective the synthetic problem had not been solved when the program was brought to a close at the end of the War" [61].

There had, however, been a synthesis. A very low level of antibacterial activity was observed in some experiments attempting a synthesis of the so-called "thiazolidine-oxazolone" structure for penicillin. By devising an elaborate system for counter-current purification of the product from such a reaction, du Vigneaud and his colleagues did obtain a minuscule yield of penicillin [26]. It was not until 1957 that a rational synthesis of the β-lactam structure, penicillin V (phenylpenicillin), was achieved at MIT by Sheehan and Henery-Logan [52].

With the cessation of hostilities, instead of attempting publication of the reports in the usual way in the open scientific literature, a massive monograph of 1094 pages, "The Chemistry of Penicillin" was prepared, and incorporated evidence and conclusions [16]. Owing to the volume of work, this multi-authored volume did not appear until 1949. It is not an easy read for a chemist of today because it uses a variety of styles for structural formulae. Moreover, unfortunately, an important structural representation of the β-lactam formula shows the biologically inactive enantiomer [5].

Penicillin and patents

As has been demonstrated here, the Bayer Company's search for an antibacterial agent was driven by the expectation of profits. Then, as now, a process that could be the subject of one or more patents was especially valuable to a pharmaceutical company. Indeed, it is reasonable to regard the entire history of the sulfa drugs as patent-driven. For example, almost the first thing one notices in the Merck Index entry for sulfapyridine (M & B 693) is that the initial reference to its preparation is to British patent 512,145 (1939) and by the same authors to US patent 2,275,354 (1942). For the penicillins, the situation is very different. The Merck Index entry for pentenylpenicillin sodium (i.e., penicillin F of Florey) notes only a US patent for crystallization of this compound; for benzylpenicillin (penicillin G) there are citations to a production process (US patent 3,024,169, 1962) and a chemical synthesis (US patent 3,159,617, 1964).

The question of a patent for penicillin had been raised by Chain during the work at Oxford. Chain's father, a chemical engineer, had organized a successful factory, Chemische Fabrik Johannisthal, in an industrial suburb of Berlin that manufactured metal salts. As a result, Chain had grown up in an atmosphere of chemical industry and research. He was aware of strong links between German academics and scientists working in industry, a situation very different from that then prevailing in the UK [15]. Chain's desire in some way to patent penicillin was a source of much discord between him and Florey. Florey discussed the situation with the Secretary of the Medical Research Council, Sir Edward Mellanby (who was responsible for providing much funding for the penicillin work) and the President of the Royal Society, Sir Henry Dale. Both of these highly placed individuals believed that it was unethical for medical research workers to profit in any way from commercial development of their work [59]. Chain himself visited Mellanby and was informed in no uncertain terms "that patenting of drugs was unethical and contrary to the traditions of medical research in Britain" [59]. In any case, as Hobby has noted, there was in the late 1930s a general agreement that a natural product, produced spontaneously in Nature, could not be the subject of a patent [33]. Only a few years later this argument was abandoned and streptomycin became the subject of a patent.

A further consideration was that in the UK until 1949 (when a new patent act was passed), although a process could be patented, a chemical could not [13]. The Oxford

process for producing penicillin had already been freely published in the literature in 1940 and 1941 [1, 14].

The dispute between Chain and the leaders of the British scientific establishment left Chain distrusting the Medical Research Council and other governmental units in the UK. It was an important factor in his decision to move to Italy where he remained for 15 years (see later).

Submerged fermentation and use of corn steep liquor

Another route to discovery involved work at a government sponsored research institute. On July 14, 1941, as part of their visit to the United States, Florey and Heatley had arrived at the Northern Regional Research Laboratory, NRRL, a unit of the United States Department of Agriculture in Peoria, Illinois. Heatley remained there until November 30 [33]. He had brought the Fleming strain of *Penicillium notatum* and a few of the glass and ceramic cylinders used at Oxford for penicillin bioassay and gave detailed instructions for their use to the staff at NRRL. Heatley worked extensively with A.J. Moyer on the development of improved cultural conditions, particularly submerged fermentation. NRRL had had experience in such techniques and had equipment available.

In Peoria, the brewer's yeast extract used in culture media at Oxford was apparently unavailable and Moyer suggested that corn steep liquor be tried as a substitute [33]. Corn steep liquor was, at that time, a waste product of the wet corn milling industry and was available in quantity. A dried preparation of the steep water had actually been patented as a "yeast food" and also found to "greatly facilitate fermentation" as long ago as 1909 by Behr [3]. In fact, concentrated corn steep liquor was used as a nutrient substitute for dried yeast extract in a semiplant-scale submerged fermentation process (approx. 550 l) for conversion of sorbitol to sorbose by *Acetobacter suboxydans*; it was described as "Yeast Compound" [58]. Although this nutrient caused excessive frothing, this was controlled by use of octadecyl alcohol. In the same way, submerged fermentation of glucose to gluconic acid by *"A. niger 3"* in rotating aluminium drums was also carried out using corn steep liquor [53] as was the bacterial fermentation of glucose to 5-ketogluconic acid (*Acetobacter suboxydans*) and 2-ketogluconic acid with an unnamed bacterium [48].

The usual penicillin literature tends to imply that the suggested use of corn steep liquor in penicillin production came de novo from the mission of NRRL to investigate uses for surplus agricultural materials. It is clear that this is an oversimplification because there was ample precedent for its use in the sorbitol and gluconic acid fermentations just noted.

The first patent application concerning penicillin production was filed at the US Patent Office on May 15, 1943 and became US Patent 2,448,790 on September 7, 1948 [28]. It was assigned to "Merck & Co., Inc., Rahway, NJ". A continuation-in-part, filed by the same authors on January 22, 1946, became US patent 2,448,791 [29]. The major claim was for the use of submerged growth conditions with aeration or aeration and agitation, thus leading to the availability "for the first time of a practical process for the large-scale commercial manufacture of penicillin". One of the examples cited in the patent involved the fermentation by *Penicillium notatum* of 600 gallons of medium in a 750 gallon tank. After five days at 20–25°C the culture filtrate "assayed 40 Florey units/ml". Using the fact that 1 mg of pure benzylpenicillin is now assigned the value of 1,650 Oxford units, the penicillin yield in such a fermentation was approximately 25 mg l^{-1} (Florey unit was an earlier term for Oxford unit). In addition to describing submerged fermentation in tanks constructed of carbon steel, the culture medium contained a significant level of corn steep liquor. The continuation patent, 2,448,791, described the use of a cottonseed meal/lactose medium [29]. In a 300-l fermentation with *Penicillium chrysogenum* X1612, the maximum activity was 381 Oxford units ml^{-1} (i.e., about 230 mg l^{-1}). This medium also contained phenylacetylethanolamine—see later.

On May 11, 1945, two years after the initial Merck application, Moyer also filed three US patent applications concerning a method for producing penicillin. They became US Patents 2,442,141, 2,443,989, and 2,476,107 and were assigned to the United States of America as represented by the Secretary of Agriculture [40–42]. Moyer referred to a co-pending application, serial number 520,234, filed on April 18, 1942 (predating the Merck application) and abandoned May 27, 1945. He noted that a wide variety of proteinaceous materials favored the production and stabilization of penicillin with corn steep liquor, "a cheap, readily available product" being especially useful. Moyer also found that it was advantageous to replace the rapidly assimilated glucose of Czapek–Dox type media with a polysaccharide; the disaccharide, lactose was eventually to become favored. In one example, a stationary submerged fermentation of 32 l of lactose/corn steep liquor medium gave a yield of 65 Oxford units ml^{-1} (approx. 40 mg l^{-1}).

Less than three weeks after filing for these US Patents, Moyer applied for three UK patents. The application date in each case was May 31, 1945, with application numbers 13674/45–13676/45; these became UK Patents 618,415, 618,416, and 624,411 [43–45]. They are very similar to his US Patents just described and much of the language is identical. In these UK patents, there is no mention of any assignment to the US government and no indication that

the work was carried out at NRRL—the only identification is to "I, Andrew Jackson Moyer, a citizen of the United States of America, of 1316 Linn Street, city of Peoria, State of Illinois, United States of America".

That Merck was able to file patents similar to those of Moyer but earlier is probably because Heatley had left Peoria early in December 1941 to do further development work on large-scale production at Merck. He remained with Merck for six months. There are contradictory reports of his experience there. Hobby claims that he was unaccustomed to a large industrial laboratory and "was often frustrated at Merck by all that went on around him and all that he was not part of" [33]. Williams, however, states that the industrial atmosphere was not uncongenial, because in a letter to Florey, Heatley noted that he might later seek industrial employment in the UK. Williams [59] also quotes a letter from Heatley to Merck (not identified) saying that "the six months I spent with Merck & Co. Inc. were the most interesting, the most agreeable and the most instructive, and I am very thankful to have had the opportunity of working for the firm".

It is ironical that the NRRL work with corn steep liquor and submerged fermentation, leading to the patents just described, involved a large assumption—that the antibiotic activity being observed was actually "penicillin". In 1941, the chemical structure of penicillin remained unknown and there was no way to identify the NRRL product with the "penicillin" made at Oxford. In both cases all that was observed was antibiotic activity. It soon turned out that the Oxford and US materials were, in fact, different and were distinguished initially as penicillin F (F for Florey) and penicillin G (next letter in alphabet after F), or as penicillin I and penicillin II. With the chemical structures being determined by 1945 [6, 16] penicillin F became pentenylpenicillin (Fig. 3a) and penicillin G, benzylpenicillin (Fig. 3b). The corn steep liquor addition had provided a precursor molecule containing the benzyl, $C_6H_5CH_2$, unit.

The eventual realization that the American workers, Foster and McDaniel at Merck and Moyer at NRRL (and later, others) had filed patents on penicillin production caused dismay in the United Kingdom. There came to be a feeling that Florey had given away the valuable secret of penicillin to "the Americans" and A.J. Moyer stood out as one of "the chief villains of the story" for his British applications as a private citizen [60]. In Britain it became widely believed that "Britain lost, or gave up, the patents on penicillin to America and has since paid many millions of dollars to US drug companies for the antibiotic. While no single item of this story is positively untrue, the whole adds up to a myth" [60].

Moyer, born November 30 1899, in Star City, Indiana, had been driven from his Indiana farm home by his stepfather at age 15 and worked his way through high school

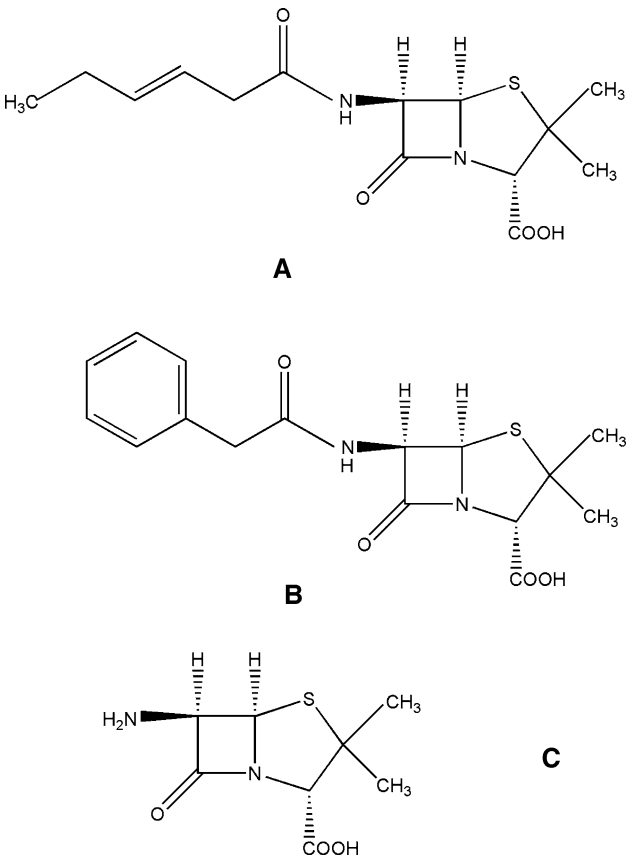

Fig. 3 Penicillins. **a** 2-pentenylpenicillin (penicillin F), **b** benzylpenicillin (penicillin G), **c** 6-aminopenicillanic acid (6-APA)

and college. He was awarded a PhD in plant pathology, University of Maryland, in 1929. He worked as a mycologist with the Bureau of Chemistry and Soils, US Department of Agriculture, from 1929 to 1940, then becoming a microbiologist at Peoria and retiring in 1957. All accounts agree that by 1941 he was a strongly anti-British isolationist with a devious and secretive nature. A Peoria staff member noted that "he was exceedingly suspicious and difficult to work with" [9]. In a photograph [33], he appears well-fed, gruff and unsmiling and seems to overwhelm an almost smiling Heatley who appears trim and slim, probably as a result of two years of wartime food rationing. Despite his isolationism, Moyer seems on the whole, to have worked amiably with Heatley. However, before Heatley moved to Merck, he had agreed with Moyer to co-author a joint paper summarizing their work. Heatley provided a draft and Moyer promised to send him a corrected version at Merck. This revision never arrived and is said to have been published later without Heatley's name [9, 59, 60]. Exactly what this paper was is unclear. However, beginning in 1945, Moyer was a co-author on a series of papers in *Journal of Bacteriology* with NRRL colleagues. The first is "Methods of Assay", co-authored by Schmidt and Moyer [50]. It refers to the second paper on

penicillin from Oxford [14], on which Heatley was a co-author, implies that it is not readily available, and in a footnote records that the authors had been advised by "the English workers" (unnamed) to use Oxford units in place of Florey units. This paper is totally devoid of any acknowledgment of Heatley for his prodigious efforts at NRRL, and there is not even a thank you. This treatment seems at best ungenerous, at worst, unethical. Later, in part VIII of that series of papers [46], Moyer and Coghill make a brusque reference to the "culture medium recommended by Dr H.W. Florey and Dr N.G. Heatley (personal communication on July 16, 1941)". They acknowledge five NRRL colleagues for help. No thanks for almost six months of effort by Heatley.

The story of the British reaction to the US and UK penicillin patents is a very tangled one; angry letters were published in "The Times" and as early as February 1944 a questioner in the House of Commons asked "whether monopolies were reducing home production of penicillin". In November 1944, another Commons speaker complained that the government "instead of taking over control of this British discovery, had handed over the manufacture almost entirely to the United States" [13]. British pride suffered with the post-war declining status of Britain compared with the United States. In addition, the immediate post-war situation proved difficult for many Britons because, despite victory in a long war, living standards actually declined. For example, bread was rationed post-war, but not in the war years themselves. As Bud [13] has noted, "penicillin can therefore be regarded as an exemplary member of a key group of British wartime inventions whose peacetime image contributed to a new sense of technocratic competence in a country whose empire was vanishing".

More questions arose in 1952, leading Vannevar Bush (Director during World War II of the Office of Scientific Research and Development) to commission a report on British allegations from John Connor, counsel for and later president of Merck [13]. The conclusion is ambiguous. No royalties for submerged fermentation had been paid by British companies but payments were made for know-how in the operation of the plants.

Wilson states that in 1945, British pharmaceutical companies such as Glaxo and Distillers bought US licenses that ran for 15 years. "There is no doubt that British companies did pay many millions of pounds in license royalties over the years" [60]. These fees were by ordinary commercial standards "modest and were slowly scaled down as penicillin prices decreased sharply at the instigation of US companies, notably Merck". It was the case that the licenses "made no limitations on British sales rights in any part of the world except America and Canada" [60]. For further detail, see Williams [59]. Before the 15-year period was complete "British companies had established a world-wide patent hold on the semi-synthetic penicillins" (see later) [60].

The price decline as penicillin became a commodity chemical [8] resulted from the many improvements in the manufacturing methods. The role of submerged fermentation and the use of corn steep liquor have already been discussed here. As the use of corn steep liquor was explored, it became clear that it contained materials ("precursors") that gave rise to the $C_6H_5CH_2CO$ group of benzylpenicillin. In fact, it was shown that addition of phenylacetic acid itself to fermentations increased penicillin yields in both surface and submerged cultures [47]. Many other precursors were subsequently identified [4, 16].

One precursor, phenoxyacetic acid, gave rise to a clinically useful, new material, developed at Lilly Research Laboratories from 1945, and termed penicillin V (phenoxymethylpenicillin). The V derives from the numbering of penicillins especially used in early work in the UK: for penicillin I and II, see earlier; penicillin III = penicillin X = *p*-hydroxybenzylpenicillin; penicillin IV = penicillin K = heptylpenicillin; penicillin V = phenoxymethylpenicillin (the group, C_6H_5–O–CH_2, replaces the group, C_6H_5–CH_2 in benzylpenicillin). Other precursors, e.g., 2-phenoxyethanol, are also used for the production of penicillin V. Penicillin V is somewhat less active than benzylpenicillin but has the advantage that it can be used orally, being more stable to stomach acid. Another interesting development at Lilly was to form a salt of benzylpenicillin with the anesthetic, procaine. This combination, procaine benzylpenicillin, reduces pain and discomfort associated with large intramuscular injections of benzylpenicillin itself. Salt formation involves the COO^- group of benzylpenicillin and the NH_3^+ group of procaine.

Another major contribution was the selection and production of high-yielding fungal strains. In this connection, there was one further episode of serendipity in the penicillin story. The high-yielding strain selected for scale up was actually discovered in Peoria, home of NRRL. A worker there, Mary Hunt Stevens, found a moldy cantaloupe at a Peoria fruit and vegetable store that yielded *Penicillium chrysogenum* NRRL 1951 [51]. It produced high penicillin yields in submerged cultures and was further improved by mutation (X-ray or UV treatment).

So where did all of this leave the villain, Moyer? One writer states (no source given) that although Moyer was justified in claiming the British patents, his seniors were most annoyed by his actions. "The US government departments involved…made sure by unofficial methods that he received no payments against these patent rights" [60].

Thus, finally, penicillin did come into the hands of industrial organizations and significant patent and monetary considerations came into play. Nonetheless, the glory

days when penicillin was validated as a most valuable antibiotic and was produced in a small, improvised pilot plant, were in an academic environment (Oxford University) and the development was driven by a small group of dedicated amateurs. They brought the supposed British talent for "muddling through" to new levels of competence. At a later date when the cephalosporins were discovered and put to use, the Oxford workers had learned the lesson and patents were obtained. This cephalosporin work is not discussed here in order to keep this article to a reasonable length.

Semi-synthetic penicillins

Another discovery mode, a collaboration between a government research institute and a pharmaceutical company, was involved in the development of semi-synthetic penicillins. In the Fall of 1948, Chain had moved to Rome, setting up a large fermentation pilot plant for antibiotics and a research department at Istituto Superiore di Sanità. One research objective was to modify the penicillin molecule so that it was resistant to the action of the enzyme, penicillinase (β-lactamase, EC 3.5.2.6). From early 1955 Chain [15] became involved with the UK Beecham group in this endeavor and also in the possible production of tartaric acid by a microbiological process. The Beecham group dated back to 1842 when Thomas Beecham had developed a number of proprietary medicines, most famously, "Beecham's Pills". These well-known pills, which were said to treat such complaints as bilious and nervous disorders, stomach wind and pain, headache, etc., etc., were essentially laxatives containing extracts of aloe. Heavily advertised, they became a household word in the UK and were available world-wide. This author remembers as a youngster, singing gleefully at Christmas-time, novel words to a familiar carol:

Hark the herald angels sing,
Beecham's pills are just the thing,
For easing pain and mothers mild,
Two for adults and one for a child.

These pills had a very long run on the market, being in use for a century and a half; they were discontinued only in 1998 [2].

To reflect a growing diversity, the manufacturer of the famous pills had, by 1955, become Beecham Pharmaceuticals Limited. In collaboration with Chain's institute, it was planned to produce, by fermentation, *p*-aminobenzylpenicillin, available by use of precursors such as *p*-aminophenylacetic acid and *p*-aminophenylacetamide. By further chemical modification of the NH_2 group on the phenyl ring it was hoped to produce new penicillins resistant to penicillinase action. While fermentation equipment was being constructed for Beecham's at Brockham Park, UK, two Beecham scientists, Rolinson and Batchelor, worked with Chain in Rome [49].

A serendipitous observation, first made in Rome, was further explored at Brockham Park, beginning in 1957, after Rolinson and Batchelor had returned to the UK. It was a simple finding with far-reaching results. When the fermentation broths producing *p*-aminobenzylpenicillin were assayed by a chemical method (hydroxylamine reaction) there was apparently a greater yield of antibiotic than indicated by a bioassay similar to that described by Heatley [15]. Surprisingly, the discrepancy was greatest when no precursor material was added to the fermentation. Meticulous work indicated that the difficulty was caused by the presence of 6-aminopenicillanic acid, 6-APA (Fig. 3c) in the fermentations—i.e., the normal β-lactam-thiazolidine nucleus lacking the usual $C_6H_5CH_2$ group found in benzylpenicillin [49]. Since the NH_2 group of 6-APA could be acylated chemically, the potential to prepare many "semi-synthetic" penicillins was apparent. Clark states that Chain compared this discovery with the finding of the activity of the sulfanilamide unit in Prontosil (see earlier) [15].

However, 6-APA was a typical zwitterion (having both NH_3^+ and COO^- groups) and was not easy to isolate from fermentation broths. Moreover, it was produced only in small amounts. A further important development in the late 1950s was the isolation of penicillin amidase enzymes (EC 3.5.1.11) from soil microorganisms and bacteria, with the ability to split off the side chain of a penicillin; e.g., to convert benzylpenicillin to 6-APA as follows:

$$\text{benzylpenicillin} + H_2O \rightarrow \text{phenylacetic acid} + 6 - \text{APA}$$

It is of interest that prior to this work at Beecham, a Japanese investigator, Koichi Kato, in 1953 had actually claimed the isolation of a penicillin nucleus (incomplete penicillin) from fermentations conducted without addition of phenylacetic acid [34]. The material was not well characterized (e.g., as 6-APA) and there was some controversy. However, Kato's claim was fully confirmed by A. Demain in 1955 [20].

The first penicillinase-resistant product formed from 6-APA was methicillin (2,6-dimethoxyphenylpenicillin). The first semi-synthetic penicillin on the market in both the USA and UK was phenethecillin (α-phenoxyethylpenicillin) [60]. Needless to say, 6-APA, methicillin, and the other semi-synthetic penicillins became the subjects of patents, the first application regarding 6-APA being filed on August 2, 1957 [60]. However, not all went smoothly with the Beecham patents. In the 1970s, an antitrust suit was filed by the US Department of Justice against three affiliated

Beecham companies, alleging that they had "combined and conspired to restrain and monopolize trade in ampicillin and other semi-synthetic penicillin drugs by fraudulent manipulation of patent rights and trade names" [57]. The case is very complex involving the disclosure of information and international law. Very unpleasant legal difficulties accrued to Chain as a result of the 6-APA work with the Beecham group [15].

Epilogue

As described here, the discovery and utilization of the sulfa drugs and the β-lactams (penicillins and semi-synthetic penicillins) were achieved variously in purely industrial locations, at universities, and at government laboratories, often by collaborative efforts. In several instances, serendipity played an important role. It cannot be claimed that any one discovery mode was superior. However, all humanity can be grateful for the efforts of the four major players, all Nobel laureates—Chain, Domagk, Fleming, Florey—and appreciative of the fact that these antibiotics, in one way or another, were put in place.

A major distinction between the two discovery processes is that the sulfa drugs did not trigger any significant new technology for production. The manufacture of Prontosil, sulfapyridine, and other sulfa drugs, required the well-established technology of chemical engineering—adapting large-scale engineering methods to chemical synthesis of materials such as dyes and medicinals such as aspirin. Indeed, the production of Prontosil can be regarded as the manufacture of yet one more azo dye.

On the other hand, large-scale production of penicillin, beginning in the 1940s, required major new technological accomplishments—development of large-capacity tanks with provision for agitation, maintenance of temperature, pH, and pure culture, and, above all, the requirement for large volumes of sterile oxygen (air). That the levels of penicillin in the fermentation broths were modest and that penicillin is a relatively unstable molecule presented major challenges. One major technological development was the development of large-scale freeze drying. All of this required the efforts not only of engineers but also of biologists. In fact, penicillin has been termed "A Paradigm for Biotechnology" [37]. Increasingly, biotechnology has involved the application of genetics exemplified early on by the development of high-yielding strains, and more recently by studies of recombinant fungal strains. The work on penicillin has provided basic information on the enzymology of biosynthetic pathways for secondary metabolite production [21].

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