

The Antibiotics—Past, Present, and Future

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The concept of antibiosis—the realization that microorganisms can synthesize chemical substances which selectively inhibit or kill other microorganisms, did not occur suddenly to Alexander Fleming, nor to any other single scientist. Like most other discoveries, this concept grew out of the cumulative experiences and observations of man dating back, no one knows how far, into the past.

It is difficult to say how far back in history we might find evidence of man's belief in the curative power of microorganisms, were we to make an exhaustive search. We know that apothecaries in the England of 1640 prized the mold that grew on dead men's skulls as an ingredient for an ointment. (1) Stories of the use of deliberately nurtured moldy bread and moldy corn are found in the folk lore of the Maya Indian centuries ago, and in this century in rural areas of the Ukraine, eastern Europe, and even in England. The story is told of an untrained technician in a 1911 laboratory of Oxford University gathering up and taking home remnants of the mold cultures used in classroom demonstrations. Asked why he did so, he said his family had used molds to treat "gatherings" for generations.

Whether these medicinal uses of molds were based on observations of forgotten origin or on superstition is unknown. But we cannot question so readily the observation by Tyndall, most noted as a physicist, who in 1876 noted that growth of a *Penicillium* mold discouraged growth of bacteria in test tubes of mutton infusion. Nor can we discount the careful description by Pasteur in 1877 of the suppression of anthrax bacilli by other common bacteria in the same medium, and the protection of small animals from anthrax infection by simultaneous introduction of other bacteria into the animal. Whether these represent actual cases of antibiotic production, we cannot be certain. We can be certain, however, that these scientists did not clearly derive the modern concept of antibiosis from their observations. Babes (1885) came much closer when he studied the antagonisms of specific bacteria for one another by methods much like the cross-streak technique used by Waksman more than fifty years later. Garré (1887) went further, showing in plate tests that the suppression of one bacterium by another was brought about by the secretion of a diffusible substance from the inhibitory organism. By 1890, several other investigators had demonstrated, by methods little different from ours today, the production of antagonistic substances. The critical flash of insight did not occur, however, and attempts to exploit the property of microbial antagonisms took the direction of therapy by clinical administration of antagonistic organisms themselves. These efforts were not significantly successful, and progress toward the use of antibacterial substances from microorganisms came to a virtual halt.

Vuillemin introduced the term *antibiosis* in 1889 as the phenomenon of one organism's actively destroying another to preserve its own life. This meaning, bearing little resemblance to present usage, was modified in 1928 by Papacostas and Gaté to very nearly the meaning we ascribe to antibiosis today. It is an interesting coincidence that this definition was

published the very year that Fleming's classic paper describing the discovery of penicillin was in preparation.

Penicillin

The discovery of penicillin, announced by Alexander Fleming in 1929 (3), is generally recognized as the beginning of a new era in medical history—an era in which chemotherapy grew from infancy to dominance in the treatment of bacterial and fungal infections in man. But while Fleming certainly called attention to the blue-green mold that synthesized penicillin, it remained for a group of Oxford University investigators ten years later to revive interest in the almost forgotten antibiotic. Fleming and the leader of the Oxford group, Dr. Howard Florey, were later awarded jointly the Nobel prize for their complementary roles in bringing to the world its first important and still in many ways its most remarkable antibiotic.

In August, 1940, the Oxford University group headed by Florey described in *Lancet* (4) their laborious partial purification of penicillin from Fleming's mold and demonstrated the effectiveness of this substance, still less than one per cent pure, in protecting laboratory animals infected with virulent streptococci, pneumococci, and *Clostridium septique*. Even in this very impure state, penicillin inhibited these organisms in the test tube at dilutions up to one part in 500,000.

Excited by this publication, I set about trying to find cultures of this mold. The Oxford paper had cited a publication of a Pennsylvania State College graduate student who had published his thesis on studies of Fleming's mold in 1935, in which he corroborated Fleming's work (5). This was Roger Reid, who was in 1940 on the staff of Johns Hopkins University, and who is now director of the Biological Sciences Division of the Office of Naval Research. I obtained a subculture of Fleming's *Penicillium* from Reid, and another from Dr. Charles Thom, Principal Mycologist in the U. S. Department of Agriculture at Beltsville, Maryland. Through the winter of 1940 and most of 1941 I experimented with *Penicillium notatum* with no spectacular success. It was not difficult to obtain activity in filtrates of the mold, but in the absence of controlled temperature, yields were low and unpredictable—usually less than one mcg per ml.

Meanwhile, the Oxford University group was working feverishly on penicillin. In August, 1941, they described in a second publication (6) their laborious accumulation, in spite of the difficulty of carrying on research in severe wartime conditions, of enough penicillin, perhaps five per cent pure, to treat a number of patients with severe infections. Their results were most encouraging in spite of the low dosages, by present standards, made necessary by the scanty supply of penicillin. In some cases, penicillin was recovered from the urine of patients, purified, and reused in order to make continued therapy possible.

By this time it became obvious to the Oxford research team that penicillin had real potential usefulness as a therapeutic agent, but that the scale of developmental work needed for adequate evaluation was impracticable in a Britain preoccupied with fighting off bombing attacks and rebuilding its defenses. In the summer of 1941, Florey and Heatley, the latter of whom was responsible for the microbiology and who had

devised the cylinder-agar plate diffusion assay now used widely in various forms, came to this country in an attempt to stimulate interest in large scale development work on penicillin.

Florey and Heatley were to see much of America before achieving their desired results (2). They first approached the Rockefeller Foundation, which had supported much of their work by grants, and were referred to the National Academy of Science. From here they were directed to see Dr. Charles Thom, eminent mycologist in the Bureau of Plant Industry. Dr. Thom took them to the top officials of the Department of Agriculture, who suggested that they might get help at the Northern Regional Research Laboratory in Peoria, Illinois. Here they found genuine interest, and here notable advances in penicillin research in this country were to be made—by N. G. Heatley, Florey's colleague who remained at Peoria for several months, and by Peoria staff members, notably K. B. Raper, A. J. Moyer, R. G. Benedict, F. H. Stodola, and others, directed by Dr. Robert D. Coghill, director of the Fermentation Division. Florey visited various industrial laboratories, among them Eli Lilly and Company, in an attempt to arouse further interest in penicillin.

Florey's reception by American industry was friendly, but lukewarm with respect to serious consideration of penicillin as a potential commercial product—and not without reason. The sulfonamide drugs had sprung to prominence from 1935 to 1941, and as the first reasonably effective chemotherapeutic agents for bacterial infections they were themselves considered miracle drugs at the time of Florey's visit. This fact along with the obvious technical problems presented by penicillin, with its strange origin and its production by the mold in yields of less than one microgram per ml, gave little cause for enthusiasm in the ranks of industrial management. To research people, of course, penicillin had considerable appeal, for there were indications that it could conceivably surpass the sulfonamides in effectiveness. The problems of inducing the mold to synthesize practical yields of penicillin, and of recovering from it a potent new drug, formidable as they appeared, presented interesting research challenges.

The result was the initiation of moderate research efforts in several industrial plants, including our own, aided by frequent progress reports from the Northern Research Laboratory at Peoria. It would be difficult to overrate the importance of the Peoria group's work to the unparalleled industrial development of penicillin that followed. It was their discovery of more effective nutrient materials to stimulate higher yields of penicillin (7), their early recognition of the potential advantages of deep tank fermentation (8), and their discovery of new *Penicillium* cultures capable of producing penicillin in deep culture (9) that brought penicillin production into the realm of economic practicability.

By late 1941, involvement of the United States in World War II caused the creation of a cooperative research program on penicillin between a number of industrial firms, universities and the Peoria Laboratories, fostered by the Committee on Medical Research of the Office of Scientific Research and Development. This cooperative effort, unprecedented both from the standpoint of size and of close cooperation of rival industrial research groups, was a rewarding and pleasant experience for

the research personnel involved. This combined effort resulted in raising penicillin yields a hundred-fold, perfecting large scale purification processes, achievement of sterile fermentations on an unprecedented scale, building of plants, and the attainment of massive production of penicillin in the span of less than two years.

Early developmental studies of penicillin were carried on chiefly by the surface culture method. Spores of *Penicillium notatum* were introduced aseptically and shaken up with the nutrient solution in flasks or bottles which were then incubated, undisturbed, for 6-10 days at 24°C. The spores germinated and formed a floating carpet of mycelium which absorbed nutrients from the shallow broth beneath it and excreted penicillin into it. When the nutrients were exhausted, the mold formed a mass of blue spores and synthesis of penicillin ceased. The solution under the mold was then filtered and assayed for antibacterial activity. Purification studies were then carried on.

Late in 1941 we cleaned out a small building that once housed rabbits on our Agricultural Research farm at Greenfield, and equipped it for maintenance of constant temperature. Our first large scale attempt to produce penicillin consisted of 300 large flat bottles, each containing one liter of nutrient broth. They were inoculated a few days before Pearl Harbor, were harvested on December 10, 1941, and the pooled filtrates contained 8 u/ml of penicillin. I remember spending long hours riding a bus to Greenfield during this period, carrying five-liter bottles of *Penicillium* spore suspensions for use in inoculating the weekly batch of bottles. There were skeptical stares from the bus passengers, for the spores were a muddy green in color, and this undoubtedly reminded them that the Greenfield laboratory was known to work with dangerous pathogens.

Early results were not aided by the fact that our incubator building was a convenient sheltered route between animal buildings for farm employees on stormy or cold days, often while we were inoculating bottles. Materials clinging to their boots and clothing were obviously rich sources of microorganisms, which had an uncanny aptitude for getting into our bottles.

Early synthetic media used by the English workers gave yields of less than 1 unit per ml, and bear in mind that a milligram of pure penicillin contains 1,667 units. Replacement of pure dextrose with crude corn sugar improved yields to 4-5 units per ml. Then the Peoria group recommended corn steep liquor as an adjuvant, and this raised our yields quickly to 40 u/ml. Replacement of glucose with lactose again boosted yields to 100 units per ml or more. This crude broth inhibited the staphylococcal test strain at dilutions as high as 1:5000.

Our first attempts at purification consisted of ammonium sulfate precipitations commonly used for toxins and proteins. The harvested filtrates were chilled overnight, $(\text{NH}_4)_2\text{SO}_4$ was added, and the precipitate was collected on filter paper by filtration. Excess moisture was pressed out between layers of blotting paper, and thus we obtained dry cakes of material that inhibited staphylococci at dilutions as high as 1:300,000. Though it contained far more $(\text{NH}_4)_2\text{SO}_4$ than penicillin, this material when taken orally produced fairly high urine concentrations of penicillin, along with diarrhea and stomach cramps, and was at least as bitter as we

used to think drugs should be. These precipitates were not suitable for systemic therapeutic use, but they provided a stable storage form, when refrigerated, for later work.

While interest in penicillin declined late in 1942, encouraging clinical success with our crude material in the treatment of carbuncles in diabetics at the Lilly Clinic in the spring of 1943 stimulated renewed interest. By this time improvements in media, higher yielding variants of the original mold, and improved fermentation conditions had also raised yields to 100-140 units per ml, more than 100-fold greater than those from which the Oxford group obtained their first meager clinical material. In the summer of 1943, the War Production Board made known its desire for all possible penicillin for use by our armed forces. A number of industrial firms made haste to get surface culture plants in operation. Late that year we were in full production in a three story warehouse converted into a penicillin factory. One floor was devoted to propagation and control laboratories, another to incubation, and the other to purification to the final product.

At peak production in 1944, 25,000-30,000 two-quart bottles were filled, sterilized, inoculated, and harvested every day, and as many as 200,000 bottles of *Penicillium* were in various stages of incubation at one time. This resulted in the daily recovery of some 6,000 liters of *Penicillium* filtrate, which at a yield of 100 u/ml would contain 600 million units of penicillin. The early purification procedure recovered, at best, 150 million units, or about 100 grams in terms of pure penicillin. The early finished product, only 10 per cent pure, was a yellow-brown powder. At worst, contamination of a few bottles with penicillinase-forming bacteria was sufficient to destroy nearly all the penicillin in the collection tank before it could be filtered.

In Peoria, Dr. K. B. Raper had screened *Penicillium notatum* and related species in the Department of Agriculture collection, and was searching for wild cultures in nature that might produce penicillin in submerged culture. By mid-1943, strains yielding 50-100 units per ml in shaken flasks and small fermentors were found and distributed to industrial laboratories. The potential advantages of submerged culture for large scale production were evident. Hence, soon after penicillin production in bottles began, plans were laid at Eli Lilly and Company for a building to house 6 eight-thousand-gallon fermentors. No precedent for such aerobic sterile fermentations existed, and Lilly had no previous experience in industrial fermentations. Nevertheless, depending upon their engineering training and what they had learned from surface culture production, J. A. Leighty, a research biochemist, now Executive Director of Scientific Research, G. B. Walden, Director of Biochemical Research, later vice president in charge of biochemical production, and S. L. McCormick, chemical engineer, designed and built a plant which began production early in 1945. It operated successfully from the beginning. One fermentor produced four times the volume of penicillin produced by the bottle plant in a week, with nearly comparable yields. The six fermentors soon raised production to 24 times the previous output.

The shift to submerged culture by most of the firms in 1944 and 1945, along with rapid improvements in mold strains and increasing technical "know-how" in a new industry resulted in fantastic increases in penicillin

production. In 1942, only 3 ounces of penicillin were made available for clinical trial in this country (2); in 1943, 29 pounds were produced; in 1944, 3,200 pounds; and in 1945, 11,000 pounds. By 1951, these figures were dwarfed by the 636,000 pound output of commercial penicillin, long since produced in crystalline form (22).

To biologists, perhaps the most interesting aspect of the boom in penicillin was the rapid improvement in mold strains. The first strain used in this way, known as NRRL 832, was found in the Peoria culture collection. Its yield of penicillin was about 50 units per ml. The search for new strains outside the laboratory soon turned up two more of promise, one from cheese, another from a cantaloupe. While the former at first looked better, a variant strain was soon recovered from the "cantaloupe" mold, which gave higher yields and at the same time proved to be highly unstable—a "mutator" (10). It was this strain, *Penicillium chrysogenum* NRRL 1951.B25, undesirable as it may have appeared, that became the ancestor of a line of increasingly high yielding strains which have in turn been used almost exclusively by penicillin producers in this country and abroad. Spores of 1951.B25 were sent from Peoria to Demerec, geneticist at the Carnegie Institute, who irradiated them and sent them to Minnesota University. Here some higher yielding mutants were selected by laboratory tests. These were sent to Wisconsin University, whose biochemistry department was equipped to test cultures in small fermentors. Among the strains provided by Minnesota University, one outstanding mutant capable of producing 500 units per ml of penicillin was found (12). The graduate student who carried out that study was Dr. J. J. Stefaniak, the present director of our Lilly Tippecanoe Antibiotics and Chemical Manufacturing plant at Lafayette (11). This strain, labeled X-1612, was used as a parent strain in an intensive development program at the University of Wisconsin. The result was a long series of *Penicillium* mutants, each with greater penicillin-producing capacity (12, 13). These have made possible tremendous gains in penicillin yields, with consequent reduction of penicillin prices from the original \$20 per 100,000 units of crude drug to a little more than ten cents per 100,000 units of crystalline penicillin in 1961.

Other Antibiotics

By emphasizing the history of penicillin so greatly, I do not mean to imply that this was the only antibiotic investigation going on in the early forties. Dubos had reported in 1939, soon after the Oxford group began work on penicillin, the discovery of gramicidin, an antibiotic produced by *Bacillus brevis* (14). While it has never attained large scale use, this antibiotic is commercially available, supplied in topical preparations and medicated gauze. Selman A. Waksman, microbiologist at Rutgers University, had long been interested in antagonisms shown by the actinomycetes—a group of soil microorganisms neglected alike by bacteriologists and mycologists. Discovery of gramicidin and the revival of penicillin undoubtedly stimulated the pace of Waksman and his graduate students. They announced in 1940 the discovery of actinomycin (15), unfortunately a toxic substance but certain forms of which have received attention recently for their antitumor activities. In 1944, Waksman and Schatz announced the discovery of streptomycin (16), which was highly

active against the tubercule bacillus. Industrial firms active in the penicillin field quickly extended their studies to streptomycin. The story of streptomycin is like that of penicillin all over again. By 1946, 3,800 pounds of streptomycin were produced for the treatment of tuberculosis. In 1954 nearly 500,000 pounds were produced in this country alone. The royalties have built a magnificent Institute of Microbiology and continue to help maintain it. Dr. Waksman was recently awarded the Nobel Prize, largely for his direction of the studies resulting in this antibiotic.

The discovery of penicillin and streptomycin stimulated an increasing volume of effort directed toward the discovery of additional new and useful antibiotics. As a result chloramphenicol was marketed in 1947, and this was followed in a single decade by chlortetracycline, oxytetracycline, erythromycin, neomycin, vancomycin, nystatin, novobiocin, amphotericin, viomycin, and a score of others. Hundreds of antibiotics have been discovered and described in the literature that have not reached the market. Many American-discovered antibiotics are manufactured in other countries along with a few discovered there. With the exception of penicillin, however, the major antibiotics were discovered and developed in the United States.

Significant in the search for new chemotherapeutic agents is the increasing attention being given to the improvement of established antibiotics by structure modification. For brevity we shall use penicillin as an example.

Penicillin was at first thought to be a single substance. Only when variables were introduced into the nutrition of the mold did it become apparent that Fleming's mold made more than one kind of penicillin. With the Oxford synthetic medium it formed chiefly penicillin, or Penicillin F; when the Peoria group enriched synthetic nutrient with corn steep liquor, benzyl penicillin or penicillin G was predominant (1). With finer analytical techniques, several other forms were found to be produced in varying amounts. Of all these, penicillin G became the commercial form because of greater ease of production and its generally greater effectiveness. It was found quite early that penicillin G yields could be markedly stimulated by the addition of phenylacetic acid to the nutrient solution (17).

Success in inducing greater penicillin G production by a precursor led to research by a team headed by Dr. O. K. Behrens, of Eli Lilly and Company, to determine whether *Penicillium* could be induced, by feeding it suitable synthetic compounds, to synthesize new forms of penicillin. The project was most successful, and more than thirty new penicillins were obtained and characterized by this method (18). One of these, commonly known as Penicillin V, was later found to be efficiently absorbed by the oral route because of its stability to acid. It has subsequently attained high repute as an orally administered penicillin.

More recently, British investigators demonstrated that penicillin-producing molds may also form the body (or nucleus) of the penicillin molecule, but lacking the accessory group that confers antimicrobial activity and the characteristics of a particular type of penicillin (19). By nutrient modifications, this inactive "nucleus," more commonly called 6-aminopenicillanic acid, can be made the predominant product. It was

an obvious step from this discovery to the isolation of the penicillin nucleus and the chemical attachment of different groups to it to form new species of penicillin. Several have now been synthesized which have promise of significant usefulness. One has the property of stability against destruction by penicillinase, and consequently is effective against destruction by penicillinase, and consequently is effective against staphylococcal strains resistant to natural penicillins (20). Another has a broader antimicrobial spectrum, though without the advantage of stability to penicillinase (21). Because these "semi-synthetic" penicillins are quite new, and since in gaining a new desirable property they may lose others or pick up new undesirable properties, it is too early to assess their true value. Other instances of useful antibiotic modifications can be cited. Tetracycline, discovered as a modification of chlortetracycline (aureomycin) is made by two methods: by inducing the aureomycin-producing organism to form tetracycline, and by chemically modifying aureomycin. Erythromycin was twice modified, first to the propionyl ester, and then to the lauryl sulfate salt of propionyl erythromycin, a compound markedly superior to the parent antibiotic.

A Look Into the Future of Antibiotics

Considering the intensity of the search for new antibiotics over the last fifteen years, the flow of new discoveries remains surprisingly steady. One factor that has helped to maintain this pace is the interest created by new applications for which antibiotics are sought. Where once only therapeutic agents for human medicine were sought, now diseases of farm animals, poultry and plants, and the potential use of antibiotics in improving animal nutrition are considered.

Actidione has long been used for certain fungal diseases of grasses and fruits, and streptomycin is used to treat a bacterial disease of orchards. A Japanese antibiotic, blasticidin, looks promising in combating a serious fungal disease of rice. Penicillin and other antibiotics of human medicine have long been used for bacterial infections of farm animals and pets. Hygromycin is used extensively in feed to eliminate and control round worm infestation of swine. Tylosin, one of the more recent antibiotics, promises to be a powerful weapon against poultry respiratory diseases caused by pleuropneumonia-like organisms. A number of antibiotics have for several years been widely used in feeds to promote more rapid, economical weight gain in farm animals. It appears certain that these early successes will sustain continued interest in the application of new antibiotics to the needs of agriculture.

But are there no new fields to conquer in diseases of man that might be susceptible to the antibiotic approach? Indeed there are. I have left them until last because the need is so great; the chances of early success, so unpredictable. I refer to the virus infections and cancer.

In spite of constant tests against viruses in connection with antibiotic screening programs, no effective therapeutic agent for the true virus infections has yet been found. Knowledge of the intimate interactions between virus and host cell is rapidly accumulating, however. In time we may be able to make a rational approach to attacking the virus after it has set up shop in its human host. One hint of possible success is represented by a substance produced by certain molds, which when admin-

istered to small animals before exposure to certain viruses, prevents infection by the virus for several days (23, 24, 25, 26, 27). This is prophylaxis rather than cure, but it may be a start toward a new approach to the suppression of viruses not yet under control—of which there are many.

As for the chances of finding microbial products for the treatment of cancer, it is too early to do more than speculate. A vast screening program involving laboratories of private foundations, government-supported laboratories, and our Lilly-supported laboratory, is devoted to the discovery and development of anti-cancer agents.

The problem is overwhelmingly complex. Cancer is not one disease, but a large family of diseases with widely different characteristics. In cancer, the problem is to attack cells for which no significant physiological differences from normal cells that might cause selective susceptibility to a drug are yet known.

No general cure for cancer has been found. A number of substances have found limited therapeutic use and often retard the progress of some types of cancer for a time. This limited success gives hope that better agents may be found.

Microbial culture filtrates are found which in small animals and in tissue culture show antitumor activity. Most of these remain to be purified and evaluated. We can only hope that some of them will be more effective than those we have seen thus far.

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