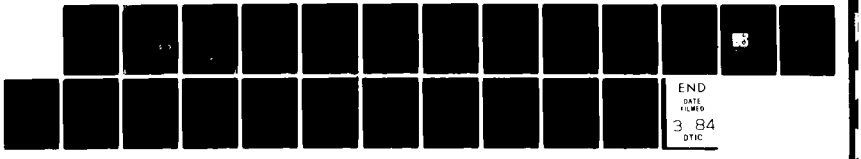
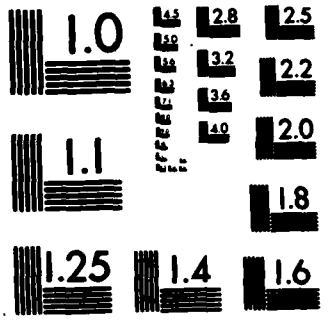
AD-A137 632 PENICILLIN UNTIL 1957(U) WALTER REED ARMY INST OF 1/1
RESEARCH WASHINGTON DC F E HAHN 1983

UNCLASSIFIED F/G 6/15 NL



END
DATE FILMED
3 84
DTIC



MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

(11)

UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A137 632	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Penicillin Until 1957		5. TYPE OF REPORT & PERIOD COVERED
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Fred E. Hahn		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Division of Communicable Disease & Immunology Walter Reed Army Institute of Research Washington, D.C. 20012		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS Project # 3M161102Bs10 Work Unit: 208
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research & Development Command Fort Detrick, Frederick, MD 21701		12. REPORT DATE 1983
		13. NUMBER OF PAGES 34
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office) Walter Reed Army Institute of Research Washington, DC 20307		15. SECURITY CLASS. (of this report) UNCLASSIFIED
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

This document has been approved for Public Release. Its distribution is unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Penicillin, Mode of Action, History of first 30 years, Review, Opening Lecture on β-Lactam Antibiotics EMBO Workshop.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Penicillin remained for 11 years after its discovery unrecognized as a chemotherapeutic drug. Early studies on its mode of action (1942) revealed that the antibiotic is only bactericidal in growing bacterial cultures. Paradoxically, low concentrations are biologically more active than higher concentrations. Penicillin causes bizarre morphological forms of exposed bacteria. It produces lysis of liquid bacterial cultures unless sucrose is added for osmotic protection. Original hypotheses that penicillin interfered with nucleic acid or

DD FORM 1473 EDITION OF NOV 65 IS OBSOLETE 84 02 08 020

UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

protein biosyntheses were erroneous.

The discovery of the accumulation of "Park's Nucleotides" gave rise to the hypothesis that the antibiotic interferes with the biosynthesis of the bacterial cell wall polymer. The binding of radioactive penicillin to bacteria could not be interpreted in terms of the mechanism of action of the antibiotic. In 1957, the prevalent hypothesis explained the bactericidal action of penicillin as a biochemical effect on the biosynthesis of the murein building block of the bacterial cell wall.

Penicillin Until 1957*

Fred E. Hahn

A. Introduction

It may seem paradoxical to include in a progress volume a contribution whose title suggests that it is retrospective and deals with the first 30 years of research on the mode and mechanism of action of penicillin. To the surprise of the author, his studies into the earlier history of this research field have brought to light a wealth of observations and experimental findings which are forgotten and no longer read. Moreover, some of this material has a distinctly contemporary ring to it.

How can this be the case? Scientists are brought up with a view of the aggregative accumulation of scientific knowledge, resembling the building of a house in which brick is mortared upon brick, each earlier structural component carrying the subsequent accretion. But in reality, things do not appear to be so simple.

Figure 1, taken from a book entitled *The Growth of Knowledge* (Kochen, ed. 1967), has been assembled by De Solla Price from data of Garfield. It depicts for each year between 1860 and 1960, the ratios of the number of citations in 1961 to the number of articles published in each year. It illustrates that during the first 20 years after publication, the bibliographical use of articles declines steeply and systematically and then continues to decline more gradually until it approaches statistically a ratio of one citation of each paper per year.

There is an "immediacy factor" in the use of published knowledge which means that about 30 per cent of all references are to the recent research front while every year about 10 per cent of all publications "die", not to be cited and reviewed again. It means that if recent work is not cited rather quickly, it may not be cited and reviewed at all, but simply be buried in the growing archive of scientific literature.

This will not only occur with articles of mediocre quality, but also with those which, in certain respects, are so far advanced that the field perceives them as "unzeitgemäss" or premature. In fact, Stent (1972) wrote an interesting study, entitled *Prematurity and uniqueness in scientific discovery.*

---

* The views of the author do not purport to reflect the position of the Department of the Army or the Department of Defense

Progress in Molecular and Subcellular Biology, Vol. 8, edited by F.E. Hahn

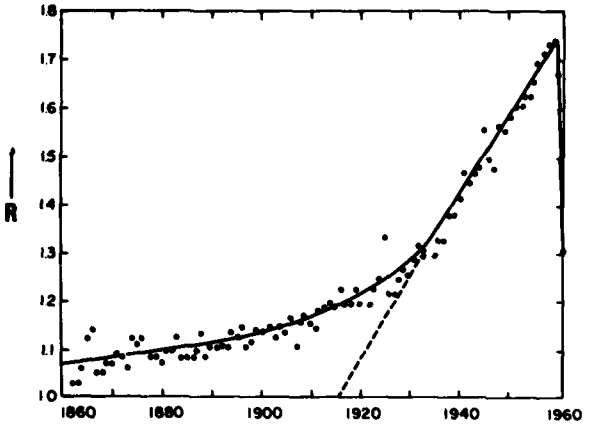


Fig. 1. Ratios of the number of 1961 citations to the number of papers published in each of the years 1860 through 1960 (De Solla Price in Kochen, 1967)

During a tenure at the University of Heidelberg, I became acquainted with the Nobel Laureate Richard Kuhn and learned that he was spending a considerable portion of his time reading the older chemical literature. As a direct result of his studies, he introduced column chromatography in 1932 which had been published 28 years earlier by Tswett and completely missed by the field. Kuhn also introduced in 1941 triphenyltetrazolium chloride as an irreversible reduction indicator, going back to an original publication of von Pechmann and Runge in 1894. The discovery of cytochromes by MacMunn in 1883 and their rediscovery by Keilin in 1925, as well as the classical genetic paper of Gregor Mendel of 1865 which was confirmed as late as 1900 by de Vries are additional examples of important articles in the source literature being overlooked and promptly forgotten.

B. The Discovery of Penicillin

For a while, this nearly happened with penicillin. The antibiotic was discovered by Fleming in 1928, although his published report dates from May 1929. I shall quote the introduction to his report (Fleming, 1929).

"While working with staphylococcus variants, a number of culture plates were set aside on the laboratory bench and examined from time to time. In these examinations, these plates were necessarily exposed to the air and they became contaminated with various microorganisms. It was noticed that around a large colony of a contaminating mould, the staphylococcus colonies became transparent and were obviously undergoing lysis."

"Subcultures of this mould were made and experiments conducted with a view to ascertaining something of the properties of the bacteriolytic substance which had evidently been formed in the mould culture and which had diffused into the surrounding medium. It was found that broth in which the mould had been grown

at room temperature for one or two weeks had acquired marked inhibitory, bactericidal and bacteriolytic properties to many of the more common pathogenic bacteria."

Fleming carried out a series of mostly bacteriological studies with the fermentation broth of the fungus. And summarizes some of them as follows:

"The active agent is readily filterable and the name penicillin has been given to filtrates of the broth culture of the mould."

"The action is very marked on the pyogenic cocci and the diphtheria group of bacilli. Many bacteria are quite insensitive, e.g. the coli-typhoid group, the influenza-bacillus group, and the enterococcus."

"Penicillin is non-toxic to animals in enormous doses and is non-irritant. It does not interfere with leucocytic function to a greater degree than does ordinary broth. It is suggested that it may be an effective antiseptic for application to, or injection into, areas infected with penicillin-sensitive microbes."

The entire paper on the discovery of penicillin does not once contain the term chemotherapy. Fleming was interested throughout his medical life in the natural antibacterial action of blood and antiseptics and makes a special point of mentioning that in vitro penicillin, which completely inhibited the growth of staphylococci in a dilution of 1 in 600, did not interfere with leucocyte function to a greater extent than does ordinary broth. By relating the action of penicillin to phagocytosis and restricting his experimentation almost exclusively to in vitro antibacterial testing he failed to call the attention of the field to the potential discovery of an antibacterial drug for systemic administration. Whereas Fleming's discovery was undoubtedly widely discussed, it was cited in the subsequent ten years no more than three times.

In 1932, three years after the discovery was published, Clutterbuck, Lovell and Raistrick published a paper which was No. 127 in a series entitled *Studies on the Biochemistry of Microorganisms.* They succeeded in fermenting penicillin from Fleming's strain in a simple mineral medium with glucose as the source of carbon, in the hope of ready isolation. But the antibacterial potency was lost during evaporation of an ether solution in a stream of air or by evaporation in vacuo at 40-45° in acid and alkaline solutions.

Three years later, in 1935, Roger Reid of Pennsylvania State College repeated the fermentation in synthetic medium. He found the antibacterial activity relatively thermo-stable but could not separate it from the rest of the filtrate by dialysis, absorption on charcoal, or distillation at low temperature.

Five years later (1940), in New York, Siegbert Bornstein used the filtrate of the penicillium cultures of Fleming's organism in studies on bacterial taxonomy. From the history of the first 10 years, it appears that the field did not appreciate the sig-

nificance of Fleming's discovery and did not rally to an intense and systematic effort of isolating penicillin, determining its structure, and trying it as a chemotherapeutic drug.

It is of interest to ask why Fleming's own work on his discovery ceased despite his appreciation of the fact that as an antiseptic, penicillin broth differed so drastically from other known antiseptics. In 1940 he wrote "We have been using it in the laboratory for over 10 years as a method of differential culture. It was used in a few cases as a local antiseptic but although it gave reasonably good results, the trouble of making it seemed not worthwhile," and one year later: "a few tentative observations had been made on the local application of the unconcentrated culture to septic wounds. Although the results were considered favourable, ... it was not considered that the production of penicillin for the treatment of these was practicable, owing to the lability of the active principle in solution." And in 1945, after penicillin as a systemic drug had become a reality, Fleming wrote: "When I saw certain changes on my culture plates as the result of the mould contaminant, I had not the slightest suspicion that I was at the beginning of something extraordinary." The three preceding quotations of Fleming are cited by Florey et al. (1949). Thus from the history of the first 12 years, it appears that the discovery of penicillin was at risk of being forgotten, like the other scientific discoveries mentioned in the introduction. This is even more surprising since sulfonamides had been introduced in 1935 and showed that substances did exist which could control systemic bacterial infections after absorption into the blood stream.

C. Penicillin as a Chemotherapeutic Drug

The first human cases to be cured by penicillin were four babies and a colliery manager. The work was carried out by Paine in 1931 but was never published in the medical literature. Only after penicillin had become famous, were these first cures discovered by investigating journalists and authors (Wilson, 1976). Paine produced his own penicillin broth. Four infants in Sheffield, two with staphylococcal and two with gonococcal eye infections were treated by infusion of penicillin broth into the eyes every 4 hours. After three days, both gonococcal infections and one of the staphylococcal infections were cured.

The colliery manager had one eye penetrated by a small chip of rock when he was down in the mine. He developed a serious eye infection with *Pneumococcus* which rendered impossible the surgical removal of the stone sliver. Fourty-eight hours of continuous irrigation of the eye with penicillin broth cured the infection, the stone chip was removed routinely, and the patient's eyesight was saved.

Dr. Paine did not continue his work with penicillin. One wonders how the history of penicillin would have developed, if these clinical results has been published. But the work went unnoticed.

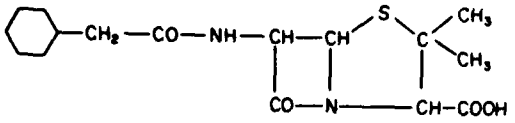


Fig. 2. Structure of Penicillin G

The decisive breakthrough came in 1940 through the work of Chain, Florey and other scientists at the Sir William Dunn School of Pathology in Oxford (Chain et al. 1940). These investigators obtained penicillin as an impure brown powder and gave their first publication the title, *Penicillin as a Chemotherapeutic Agent*. They ascertained the non-toxicity of penicillin solutions injected into laboratory rodents and showed that the drug was active in vivo against three Gram-positive organisms against which it had shown activity in vitro. One year later there followed a long paper (Abraham et al. 1941) on a therapeutic trial involving 10 cases of human infections: five patients with staphylococcal and streptococcal infections were treated intravenously, a baby with a persistant staphylococal urinary infection, by mouth and four cases of eye infections by local application. In all cases, a favorable therapeutic response was obtained.

In the same year, 1941, a paper was read by Dawson, Gladys Hobby, Karl Meyer and Chaffee before the Annual Meeting of the American Society for Clinical Investigation, entitled *Penicillin as a Chemotherapeutic Agent* in which inter alia it was reported that penicillin protected mice against 100,000,000 lethal doses of hemolytic streptococci. Hence, in 1941, Penicillin had been put on the map of chemotherapeutic drugs.

Despite these early successes in characterizing penicillin as an extraordinarily potent chemotherapeutic drug, two important items of scientific information were still missing. The drug had not been purified from fermentation mixtures, and its chemical structure had not been determined. By 1943, the recognition of the potential medical importance of penicillin resulted in the restriction of information on its chemical nature. Although some 40 chemical laboratories in the United Kingdom and the United States worked on penicillin, their results were exchanged and communicated only in the form of internal reports, and the first brief summaries of the results appeared in print in Nature (1945) and in Science (1945) after the cessation of hostilities.

D. Early Studies on Mode of Action

While the confidential work on the purification, structure, and derivatization of penicillin was under way, a first study on the mechanism of action of the crude antibiotic was published in 1942 by Hobby, Meyer and Chaffee. Penicillin was found to be bactericidal for Gram-positive cocci, and the rate of killing of the bacteria was of first order with time. There was a limit beyond which an increase in the concentration of penicillin did not accelerate the rate of killing. The authors did not observe

bacterial lysis, and the amount of penicillin in 48 h cultural filtrates was undiminished.

Most important, penicillin only killed bacteria under conditions which permitted the growth of control cultures. This observation that active bacterial growth was required for the bactericidal action of the antibiotic was made repeatedly in subsequent studies. Today, the interpretation of this finding would be that the drug gives rise to some form of unbalanced and lethal biosynthesis.

E. Paradoxical Inhibitory Zone Phenomena

When a well containing penicillin solution or a paper disc impregnated with penicillin are placed on a culture plate, seeded, for example, with *Staphylococcus aureus*, the zone phenomenon, depicted in Fig. 3 is typically observed (Pratt and Dufrenoy 1949). Close to the source of penicillin is a zone of growth inhibition, the diameter of which is, within limits, a function of the concentration of penicillin under test. The larger part of the plate exhibits normal bacterial growth. At the boundary of the zone of inhibition, however, a ring of enhanced growth of the bacterial population is to be seen. This phenomenon is reproduced easily and suggests the existence of a critical threshold of penicillin concentration below which it is not growth E_{max} inhibitory but, in fact, stimulates bacterial growth.

Staining of these culture plates with redox indicators revealed that the rings of enhanced growth exhibited very strong reductive activity, but the subsequent literature has failed to yield a biochemical explanation of the paradoxical zone phenomenon.

Penicillin is not the only growth inhibitor which gives rise to paradoxical zones of growth stimulation. The same phenomenon has

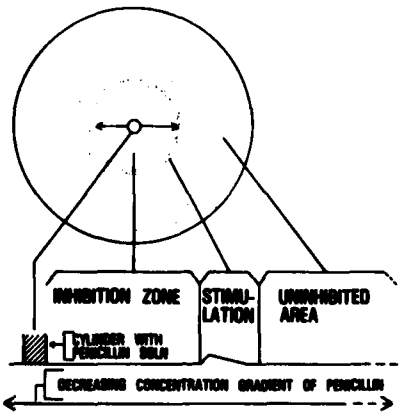


Fig. 3. Diagrammatic representation of a penicillin assay plate, showing ring of enhanced growth of *Staphylococcus aureus* on uniformly seeded culture plates supplied with a cyclinder of penicilling solution: surface view above, sectional view below. (Pratt and Dufrenoy 1949)

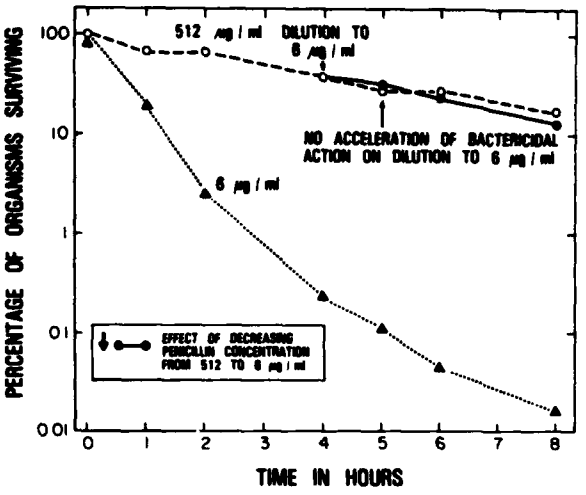


Fig. 4. The persistent effect of brief exposure to high concentrations of penicillin on the death rate of *Streptococcus faecalis* (Eagle 1951)

been demonstrated for sulfanilamide and for mercuric chloride (Lamanna and Shapiro 1943).

An obverse zone phenomenon in the bactericidal action of penicillin has been demonstrated in liquid culture (Eagle and Musselman 1948; Eagle 1951). It was shown that certain strains of bacteria are killed more rapidly by low concentrations of penicillin than by high concentrations. When such zone-reacting bacteria are first exposed to optimal, i.e. low concentrations of penicillin at which they die rapidly and subsequently high concentrations of penicillin are supplied to the cultures, the rate of death is immediately retarded to that characteristic for higher concentrations.

The paradoxical slowing down of the bactericidal effect by high concentrations of penicillin persists after the removal of the drug (Eagle 1951). If zone-sensitive bacteria are first exposed to high concentrations of penicillin at which they die at a slow rate, and the mixture is diluted after several hours incubation to the maximally effective low level of penicillin, the rate of death is not accelerated to that characteristic for the lower penicillin concentration, but the organisms continue to die at the slow rate initially established by the high concentration. Exposure to high concentrations for as short as 15 minutes, i.e. before an appreciable number of bacteria has been killed, suffices for the subsequent protection against rapid killing by low penicillin concentrations.

F. Morphological Changes in Bacteria

Beginning with the original observation of Gardner (1940), a considerable literature (reviewed by Florey et al. 1949) described the bizarre morphological changes, caused by the action of penicillin, frequently at sub-inhibitory concentrations as small as one tenth the amount required for complete growth inhibition. Large forms of irregular shape were observed in Gram-positive and Gram-negative species. Cocci produced swollen forms and bacilli formed long filaments. These malformations were attributed by Gardner to an interference with the fission of multiplying cells.

Morphological changes caused by penicillin included the formation of L-forms (Bringmann 1952; Lederberg 1956). Such penicillin-induced L-forms can revert to normal morphology when cultivated in the absence of the antibiotic (Johnstone et al. 1950; Lederberg and St. Clair 1958).

Perhaps the most important article on penicillin-induced morphological changes was published by Duguid in 1946 which remained unnoticed for 10 years. A series of sensitive and relatively resistant bacteria was grown on blocks of nutrient agar which incorporated different concentrations of penicillin and which were mounted between a slide and coverslip under an incubated microscope.

Figure 5 shows the effect of different concentrations of penicillin on the growth of *E. coli* observed over a graded period of time. "Up to the stage of filament formation and swelling, the abnormal cells were apparently alive, for growth had been proceeding and normal motility was exhibited in the case of the motile strains. Lysis, and thus death, of the filamentous cell was in most cases initiated by the gradual or sudden protrusion of one or sometimes more bubbles of protoplasm; following this, the filament became pale or even disappeared entirely. Some filamentous cells underwent lysis without any visable protoplasmic protrusion, and some without even having developed a swelling."

"The morphological changes described above as failure of proper cell division and the ready occurrence of swelling and protoplasmic protrusion, suggest that penicillin in these concentrations interferes specifically with the formation of the outer supporting cell wall, while otherwise allowing growth to proceed until the organism finally bursts its defective envelope and so undergoes lysis. In the higher concentrations, penicillin must act somewhat differently."

The significance of this 1946 publication is that it postulated on purely morphological grounds the theory of penicillin as an inhibitor of bacterial cell-wall biosynthesis which was suggested on biochemical grounds in 1957 by Park and Strominger and dominated the thinking about the mechanism of action of penicillin in subsequent years.

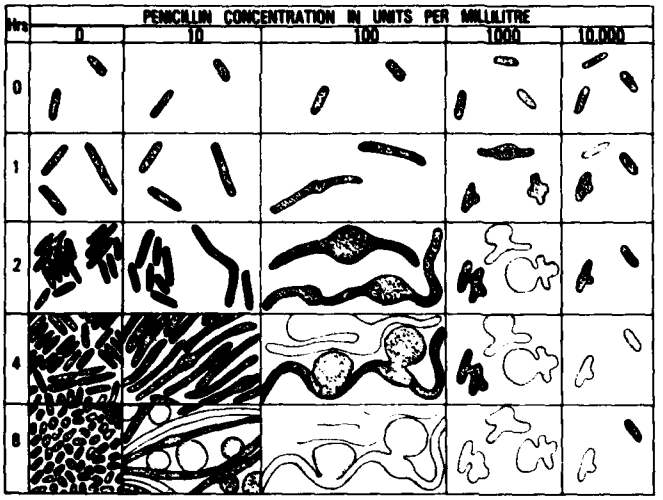


Fig. 5. Morphological effects of penicillin on growing *Escherichia coli* (Duguid 1946)

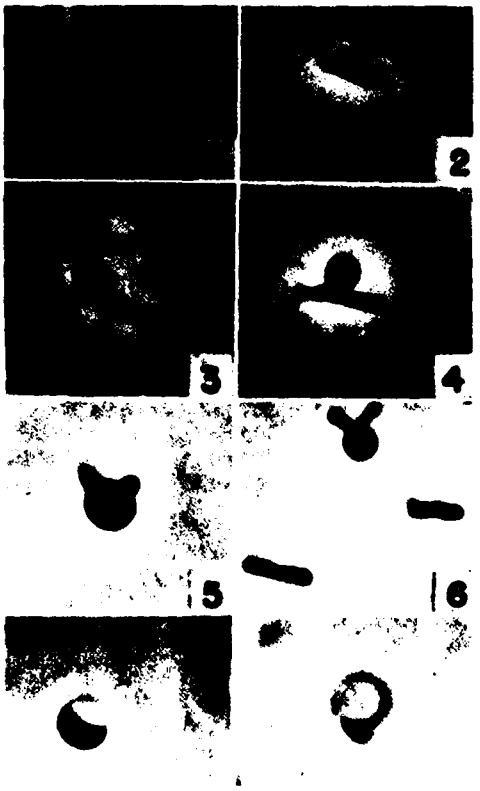


Fig. 6. Morphological effects of penicillin on *Escherichia coli*, growing in liquid medium with 0.48 M sucrose for osmotic protection (Hahn and Ciak 1957)

In 1956 and 1957, several groups of authors studied penicillin-induced changes in bacterial morphology in liquid cultures. Liebermeister and Kellenberger (1956) worked with *Proteus vulgaris* and obtained a systematic transition of bacillary into globular forms, especially when penicillin was added at the end of the exponential phase of growth. Earlier addition of penicillin produced lysis of the these cultures.

Lederberg (1956) and Hahn and Clark (1957) studied *E. coli* cultures to which sucrose had been added for osmotic protection. Figure 6 shows the sequence of morphological changes of *E. coli* B photographed under the phase-contrast microscope in my laboratory. The bacterial rods produced central or terminal globular extrusions that increased in size while the bacterial cell walls became correspondingly empty of cytoplasm. Later the globes either separated from the cell walls or retained parts of them attached, giving a typical rabbit-ear appearance. Finally, the globular structures underwent partial vacuolization, showing many crescent-shaped forms. Eventually, they released their entire content, leaving as formed elements only circular "ghosts" that probably represented empty cytoplasmic membranes."

G. Bacterial Lysis by Penicillin

While the original discovery of Fleming concerned the lysis of fully grown cultures of *Staphylococci* by penicillin, elaborated by a mold culture, the years 1943-1946 saw the publication of a rather extensive literature on the progress of lysis in liquid cultures of *Staphylococcus* which was followed turbidimetrically.

Figure 7 from a paper of Chain and Duthi (1945) shows the typical result of this experimental effort. There was general agreement that the turbidity of a young culture in nutrient medium, containing penicillin, first increased and then gradually decreased until bacterial lysis was complete. The initial increase in turbidity was alternately interpreted as being due to multiplication of the bacteria or as the result of swelling of staphylococci before lysis. Chain and Duthie compared their turbidimetric measurements with the total cell counts and showed that there was no increase in the number of cells.

In 1957 after a hiatus of more than 10 years, Hahn and Ciak and Prestidge and Pardee published results on the penicillin E_{max} induced lysis of *Escherichia coli*. The first two authors correlated the morphological destruction of the bacteria with turbidimetric measurements of lysis.

In the absence of sucrose for osmotic protection, turbidity of liquid cultures slightly increased during the first hour of penicillin action and then rapidly decreased. Aerated cultures began to foam, and masses of macroscopic long strands appeared that gave the impression of highly polymerized material. Perchloric acid extracts of such collected strands had absorption spectra resembling those of nucleic acids and contained quantities of

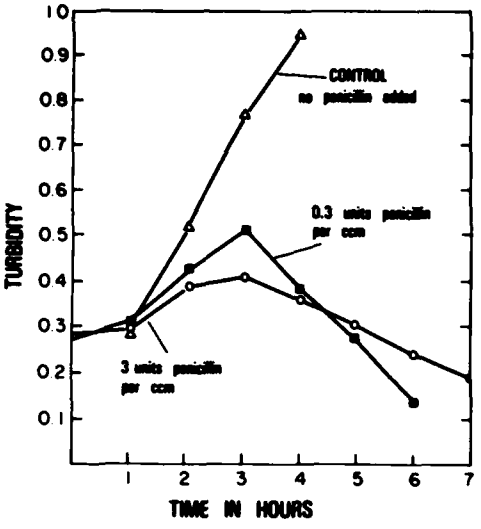


Fig. 7. Penicillin-induced lysis of *Staphylococcus*, growing in liquid medium (Chain and Duthi 1945)

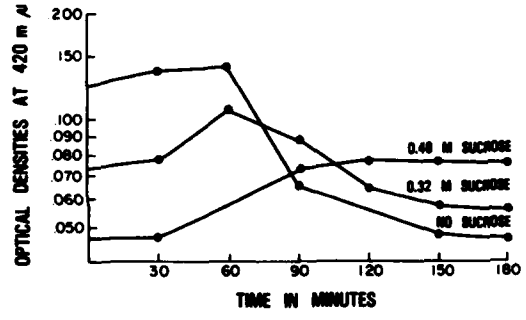


Fig. 8. Penicillin-induced lysis of *Escherichia coli* growing in liquid media with and without sucrose for osmotic protection (Hahn and Ciak 1957)

pentose and deoxypentose which suggested the presence of RNA and DNA in a ratio of 3.5 to 1.

Somewhat slower lysis occurred in the presence of 0.32 M sucrose, but a sucrose concentration of 0.48 M produced a turbidity increase that levelled off after 2 h. Samples from this culture were taken at 30 min intervals and photographed under the phase microscope to demonstrate the sequence of morphological events shown in Fig. 6.

Penicillin-induced lysis of *E. coli* occurred only in a nutritional environment that was capable of supporting bacterial growth. Suspensions of bacteria in media devoid of sources of carbon or nitrogen did not undergo lysis in the presence of penicillin.

Prestidge and Pardee (1957) refined this observation by showing that chloramphenicol, which is a specific inhibitor of protein biosynthesis, protected *E. coli* from the action of 150 μg/ml peni-

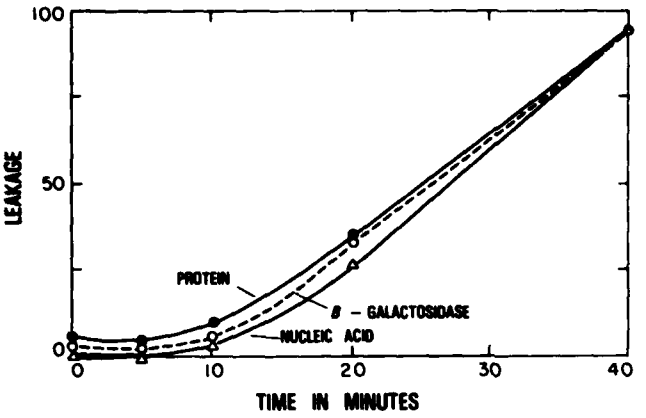


Fig. 9. Release of protein, β-galactosidase and nucleic acids from penicillin-exposed *Escherichia coli* (Prestidge and Pardee 1957)

cillin. When chloramphenicol at 20 µg/ml was supplied at various times to penicillin-containing cultures, the number of bacteria saved from the bactericidal action of penicillin decreased systematically from 52 per cent with chloramphenicol, added at zero time to 12 per cent with chloramphenicol added 20 minutes after penicillin. This work was based on earlier studies of Jawetz et al. (1951) on the interference of chloramphenicol with the action of penicillin.

Prestidge and Pardee did not only present time curves showing the loss of RNA and protein from penicillin-exposed *E. coli*, but also demonstrated the leakage from the bacteria of protein, β-galactosidase and nucleic acids into the experimental medium as a function of time (Fig. 9). They briefly discussed the possbility of a direct action of penicillin on the bacterial membrane, but considered this unlikely because of the observations of Lederberg and Hahn and Ciak which showed that osmotically protected bacteria are converted by penicillin into protoplasts with morphologically intact membranes which in Lederbergs work, after removal of penicillin could partly revert to normal bacillary forms.

H. Interference of Penicillin with Nucleic Acid and Protein Synthesis?

Krampitz and Werkman (1947) and Gros and Macheboeuf (1948) showed that in washed suspensions of certain bacteria, inhibition of RNA dissimilation was caused by concentrations of penicillin 1000 or 10,000 times greater than growth-inhibitory concentrations. Following this, Mitchell and Moyle (1951) proceeded to demonstrate certain imbalances in nuclear acid and free nucleotide and nucleoside composition in *Micrococcus pyogenes* exposed to 50 µg/ml of penicillin in growth medium. These imbalances will not be described in detail. The conclusion is warranted that penicillin does not exert a direct and primary effect on the metabolism of nucleic acids.

For a number of years, beginning in 1947, the idea was entertained that penicillin interfered indirectly or directly with bacterial protein biosynthesis. Gale and his co-workers (Gale and Taylor 1947) reported that Gram-positive bacteria were able to assimilate glutamic acid from the medium in which they were grown and to concentrate this free amino acid within the bacterial cell. They also reported that when certain strains of *Streptococcus faecalis* and *Staphylococcus aureus* were exposed to penicillin during exponential growth, the ability to concentrate free glutamic acid was lost. Gale therefore suggested that "penicillin interferes with the mechanism whereby certain amino acids are taken into the cell, and that the sensitivity of the cell to penicillin is then determined by the degree to which its growth processes are dependent upon assimilation of preformed amino acids rather than upon their synthesis."

However, in 1949, Hunter and Baker obtained a strain of *Bacillus subtilis* which grew readily in a synthetic medium which contained only ammonium sulfate as a source of nitrogen. In this medium, the organism was just as sensitive to penicillin as it was in organic media such as tryptose phosphate broth. They concluded that penicillin inhibited the growth of this strain of *B. subtilis* by some mechanism other than interference with the assimilation of preformed amino acids by the bacterial cell.

One year later, Hotchkiss (1950) published a paper on the abnormal course of bacterial protein synthesis in the presence of penicillin. Washed normal staphylococcal cells, respiring in solutions containing glucose and various mixtures of amino acids, utilized the amino acids and showed an increase in the cellular protein nitrogen. Exposure to penicillin G permitted utilization of oxygen, phosphate and amino acids at essentially the control rates, but there was no increase in the protein nitrogen of the cells. Instead, penicillin-treated cells produced increasing amounts of extracellular substances containing non-amino nitrogen in quantities approximately equivalent to the amino acid nitrogen utilized. This extracellular fraction was tentatively identified as a tetra- or pentapeptide which was produced instead of cellular protein when penicillin was present. Hotchkiss interpreted his observations as indications that penicillin interfered with the conversion of amino acids into staphylococcal protein, i.e. trichloracetic acid insoluble material, in such a manner that extracellular peptides are formed.

Shortly thereafter, Gale and Folkes (1953) discovered that the incorporation of certain amino acids into the trichloracetic acid-insoluble fraction of *S. aureus* was inhibited to the extent of approximately 60 per cent by growth-inhibitory concentrations of penicillin. The inhibition was unusual in that only certain amino acids were affected and that the levels of inhibition reached plateaus which were different for each amino acid. Much later, it became apparent that the amino acids concerned, viz., glycine, glutamic acid, lysine, and alanine are those which occur in the mucopeptide of the bacterial cell wall. So, in fact, Gale, and probably Hotchkiss, were registering amino acid incorporation into the bacterial cell-wall building blocks, and

this was, for a while, mistakenly interpreted as an interference with bacterial protein biosynthesis.

I. Park's Nucleotides

Up to this point, I have reported a considerable volume of observations and experimental studies on the action of penicillin as well as various hypotheses. But this entire material did not lead to the recognition of the molecular mechanism of the target reaction whose inhibition is responsible for the bactericidal effect of penicillin. No biochemical reaction of vital importance to the bacterial cell had been demonstrated to be inhibited by bactericidal concentrations of the antibiotic.

It should be remembered that biochemistry during the second part of the 1940's was much concerned with the phosphorylation of metabolic intermediates which was aided by a chapter of Le Page and Umbreit entitled *Methods for the Analysis of Phosphorylated Intermediates* (Umbreit et al. 1945).

Two years later, Park began his studies on the action of penicillin. The original question asked was whether the increase in size of *Staphylococcus aureus*, growing in the presence of 0.1 µg of penicillin, represented an actual increase in cell mass, and especially, whether the various constituents of the bacteria increased at comparable rates (Park and Johnson 1949). The result of the study was that the acid-soluble organic phosphate content of the cells increased at an accelerated rate under the influence of penicillin. This phenomenon was accompanied by a similar increase in a pyrimidine base, tentatively identified as uracil, an increase of pentose identified by the orcinol method, and an increase in reducing material. Measured by dry weight, of nitrogen, phosphorus, and nucleic acid, the cell substance increased by almost 50 per cent in the presence of penicillin. It was inferred that the labile phosphate compound represented a new form of organically bound labile phosphate related to the hypothetical reaction, inhibited by penicillin. The question remained somewhat open whether the reducing material, the material with an absorption maximum of 262 nm, and the labile phosphate were all constituents of a single compound.

One year later a Federation Abstract (Park 1950) followed. It reported that the unknown material was resolved by counter-current distribution into three components. Each of the separated components contained in equimolar proportions uracil, labile phosphate, stable phosphate, pentose and an unknown sugar. The preparation most soluble in the phenol phase contained 3 moles of alanine per mole of labile phosphate and probably one mole of glutamic acid. The second component contained only alanine, while the third component contained no amino acids. The three compounds became known as Park's nucleotides and it was suggested that this series of molecules represented normal intermediates of the cell metabolism and that the inhibition of a specific but

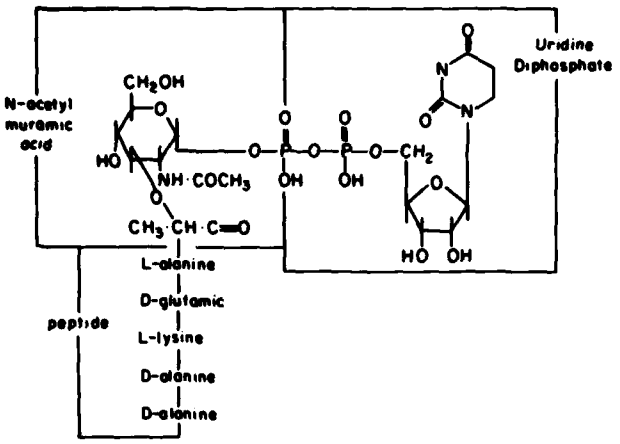


Fig. 10. Park's nucleotide, containing 5 amino acids (Park 1952c)

unidentified reaction by penicillin caused them to accumulate in abnormal amounts.

In 1952 there followed the final series of three companion papers by Park. The first (1952a) presented in detail the isolation of the three compounds from penicillin-exposed *Staphylococcus aureus* and evidence that all three substances were uridine-5-pyrophosphate derivatives. The second paper (1952b) elaborated on the structure common to all three nucleotides, and the third (1952c) contained evidence that the first compound contained no amino acids, the second compound contained one L-alanine residue while the third compound contained a peptide composed of L-lysine, D-glutamic acid and three alanine residues which were a mixture of L-alanine and D-alanine.

Figure 10 shows the definitive structure of the third nucleotide of Park. The structure of the N-acetyl amino sugar was elucidated by Strange (1959), and the final assignment of the stereochemical configurations of the amino acids in the peptide moiety was made by Strominger after the cut-off year of this review.

Until 1957 the role of Park's nucleotides in cell metabolism and in the action of penicillin remained cryptic, although a scholarly lecture by Mitchell (1956) offered a one-sentence speculation that "it is possible that these compounds may be involved in cell envelope synthesis."

The final breakthrough came in 1957, in an article by Park and Strominger, entitled *Mode of action of penicillin: Biochemical basis for the mechanism of action of penicillin and for its selective toxicity*, published in Science (1957). It reviewed the structures of Park's nucleotides and proposed a structure, as shown in Fig. 10, in which only the stereochemical configuration of the three alanine residues remained unspecified. The paper called attention to the fact that the rate of accumulation of the peptide-containing uridine nucleotide in the presence of penicillin suggested that the antibiotic inhibited one of the principal biosynthetic reactions of the bacterial cell.

The paper then reviewed what was known, from the work of others, about the chemical composition of the cell wall and suggested that the uniqueness of the structures in the wall and in the nucleotide means that they must be metabolically related. The reasoning culminated in the conclusion that the accumulation of this compound in penicillin-exposed *Staphylococcus aureus* is a consequence of the interference by penicillin with the biosynthesis of the cell wall. Uridine pyrophosphate glycosyl compounds were regarded as activated intermediates, and the N-acetylamino sugar peptide was regarded as a nucleotidyl fragment, activated for cell wall polymer synthesis. This conclusion was reached 11 years after Duguid had assumed, on the basis of morphological observations, that penicillin was an inhibitor of the formation of the outer supporting cell wall.

Park and Strominger declined to speculate on the exact nature of the interference by penicillin except by referring to the formation of protoplasts as evidence that penicillin interferes with the maintenance of the cell wall or with its synthesis. Duguid had suggested that, except for cell wall biosynthesis, bacterial growth proceeds until the organism finally bursts its defective envelope and so undergoes lysis.

In the same year, 1957, Lederberg in a one-page note expressed some careful doubts in the one-target hypothesis of the action of penicillin. He introduced his comments with a quotation from Eagle and Saz of 1955 "The mechanism whereby penicillin exerts its cytotoxic effect remains obscure." Lederberg emphasized that penicillin-induced bacterial protoplasts, when maintained in an osmotically protective medium, revert to colony-forming bacillary forms when diluted in protective growth media, lacking penicillin. He thought that more remote influences on cell wall formation than those observed by Park and Strominger cannot be precluded and suggested that further studies of antibiotic effects must be conducted with protected protoplasts rather than with lysed or lysing cells in which the ramification of secondary lesions is an inevitable complication."

J. Penicillin Binding by Bacteria

The final section of this review deals with the binding of radioactive penicillin to bacteria and bacterial fractions. This work was started in 1948 by Rowley and his associates and was still in progress when it was briefly and factually reviewed by Eagle and Saz (1955) and discussed in great detail by Cooper (1956).

It is intuitively obvious that in order to affect a living cell, antibiotic molecules must be able to reach and to interact with a vitally important cellular system, the binding site being considered the site of action of the drug. It may represent the molecular machinery operating the biochemical reaction originally inhibited or disorganized by the drug. The study of drug binding to their sites of action is an indispensible part of the investigation of the mode or mechanism of drug action.

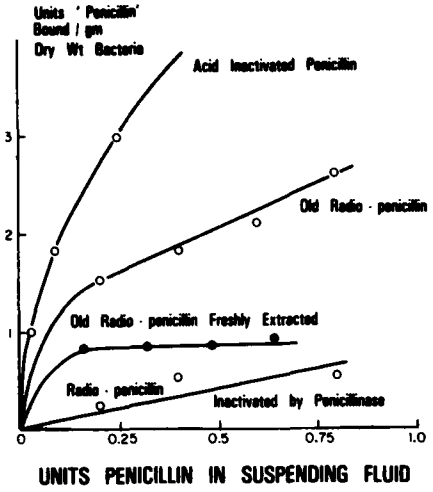


Fig. 11. Bacterial binding of penicillin and degradation products (Cooper et al. 1954)

Penicillin-sensitive strains of bacteria rapidly bound and concentrated the antibiotic with nearly complete equilibration within 1 h at 37°C. With wild strains of bacteria, such as the highly sensitive *Streptococcus pyogenes*, the amount of penicillin bound at concentrations of 0.001-0.1 µg/ml was concentrated up to 200-fold. The binding was specific. It was not observed with penicillamine or penicilloic acid. It also was irreversible. Bound radiopenicillin could not be displaced by fresh non-radioactive penicillin. Extensive washing or treatment with anionic, cationic, or neutral detergents did not liberate bound radiopenicillin. Likewise, pretreatment with such detergents did not affect subsequent binding but pretreatment with non-radioactive penicillin precluded subsequent binding of radiopenicillin. Schepartz and Johnson (1956) reported that alkaline treatment of *Micrococcus pyogenes*, treated with radiopenicillin, resulted in the cleavage of penicilloic acid from the bacteria.

In addition to the specific binding of penicillin, there was non-specific binding of penicillin degradation products. Figure 11 is from a paper of Cooper et al. (1954). The top curve shows the binding by *Staphylococcus aureus* of acid-inactivated penicillin. The second curve, marked old radiopenicillin, demonstrates the progressive binding of ^{35}sulfur from labelled penicillin preparations that had been stored. When this material was repurified by extraction into chloroform at pH 2.3 and back into neutral buffer, the saturation curve through the black dots was obtained. Finally, when this freshly purified radiopenicillin was hydrolyzed with penicillinase, the bottom line was seen. The use of purified radiopenicillin indicated that saturation of the bacterial binding sites was attained at 0.1 µg/ml.

In the same year, 1954, Eagle published an extensive study of radiopenicillin binding to five different bacterial strains. This work used C^{14}- or S^{35}-labelled penicillin. He confirmed that the amount bound from low concentrations was related to the peni-

cillin sensitivity of the strain. Despite wide differences in their sensitivity to penicillin the antibiotic was bound at biologically equivalent levels of 99.9 LDs in comparable amounts. The lethal intracellular concentrations ranged from 1.7 to 4 µg/ml, i.e. 1600 to 3300 molecules penicillin per cell.

Bacteria in the logarithmic phase of growth or suspended in salt solutions, or extracts of bacteria prepared by sonic oscillation had approximately the same reactivity with penicillin. That is, penicillin binding was independent of the metabolic state of the bacteria and was not influenced by differences in the permeability of the cells.

However, at high concentrations of penicillin from 1 to 1000 µg/ml, non-specific additional binding by all the bacterial strains and extracts was observed. This was unrelated to their sensitivity. At these high concentrations, penicilloic acid was bound to the same extent as penicillin itself.

The penicillin binding studies up to the mid 1950's logically led to the asking of two questions, the answers to which remained elusive. The first question concerned the chemical properties of the penicillin-binding component of bacteria. Despite a detailed discussion of experimental studies, Cooper (1956) conceded that "little success has been obtained in characterizing this component, and nothing is known with certainty of its chemical nature." The possibility was considered that the penicillin binding component was located in the osmotic barrier, which is cytologically observed, under the cell wall.

The second question concerned the role of penicillin binding in the antibacterial effects of the antibiotic. It has already been mentioned that Eagle found bacteria to combine with amounts of penicillin far in excess of those which are bound at the bactericidal concentrations. Bacteria suspended in salt solutions combined with penicillin to the same degree as organisms in the logarithmic phase of growth. When such treated bacteria were resuspended in penicillin-free growth medium, they eventually resumed multiplication at normal rates without any release of bound penicillin. It followed that the binding of penicillin alone did not suffice to initiate the bactericidal effect. It is necessary that the cell be in a medium which permits active metabolism and growth and that the antibiotic be continuously present in the surrounding growth medium.

To sum up: during the period on which I have reported, the biochemical theory of the inhibition of cell wall biosynthesis by penicillin emerged as the logical explanation of the antibiotic's mechanism of action but it was not possible to connect this body of knowledge and thought with the experimental results of penicillin binding studies.

References

Abraham, E.P., Fletcher, C.M., Florey, H.W., Gardner, A.D., Heatley, N.G., Jennings, M.A.: Further observations on penicillin. Lancet 177-188 (1941)

Bornstein, S.: Action of penicillin on enterococci and other streptococci. J. Bacteriol. 39, 383-387 (1940)

Bringmann, G.: Elektronenmikroskopische Beobachtungen der Entstehung filtrierbarer (L-) Formen von B. proteus unter Penicillin-Einfluss. Z. Hyg. Infektionskrankh. 135, 557-565 (1952)

Chain, E., Duthie, E.S.: Bactericidal and bacteriolytic action of penicillin in the staphylococcus. Lancet 652-657 (1945)

Chain, E., Gardner, A.D., Heatley, N.G., Jennings, M.A., Orr-Ewing, J., Sanders, A.G.: Penicillin as a chemotherapeutic agent. Lancet 226-228 (1940)

Clutterbuck, P.W., Lovell, R., Raistrick, H.: CCXXVII. Studies in the biochemistry of micro-organisms. XXVI. The formation from glucose by members of the *Penicillium chrysogenum* series of a pigment, an alkali-soluble protein and penicillin — the antibacterial substance of Fleming. Biochem. J. 26, 1907-1918 (1932)

Cooper, P.D.: Site of action of radiopenicillin. Bact. Rev. 20, 28-48 (1956)

Cooper, P.D., Clowes, R.C., Rowley, D.: A note on the use of radioactive penicillin. J. Gen. Microbiol. 10, 246-249 (1954)

Dawson, M.H., Hobby, G.L., Meyer, K., Chaffee, E.: Penicillin as a chemotherapeutic agent. J. Clin. Invest. 20, 434 (1941)

Duguid, J.P.: The sensitivity of bacteria to the action of penicillin. Edinburgh Med. J. 53, 402-412 (1946)

Eagle, H.: Further observations on the zone phenomenon in the bactericidal action of penicillin. J. Bact. 62, 663-668 (1951)

Eagle, H.: The binding of penicillin in relation to its cytotoxic action. I. Correlation between the penicillin sensitivity and combining activity of intact bacteria and cell-free extracts. J. Exp. Med. 99, 207-226 (1954)

Eagle, H., Musselman, A.D.: The rate of bactericidal action of penicillin in vitro as a function of its concentration, and its paradoxically reduced activity at high concentrations against certain organisms. J. Exp. Med. 88, 99-130 (1948)

Eagle, H., Saz, A.K.: Antibiotics. Ann. Rev. Microbiol. 9, 173-226 (1955)

Fleming, A.: On the antibacterial action of cultures of a penicillium with special reference to their use in the isolation of *B. influenzae*. Brit. J. Exp. Pathol. 10, 226-236 (1929)

Flory, H.W., Chain, E., Heatley, N.G., Jennings, M.A., Sanders, A.G., Abraham, E.P., Florey, M.E.: Antibiotics, Vol. II. Oxford: University Press 1949

Gale, E.F., Folkes, J.P.: The assimilation of amino acids by bacteria. 15. Actions of antibiotics on nucleic acid and protein synthesis in *Staphylococcus aureus*. Biochem. J. 53, 493-498 (1953)

Gale, E.F., Taylor, E.S.: The assimilation of amino-acids by bacteria. 5. The action of penicillin in preventing the assimilation of glutamic acid by *Staphylococcus aureus*. J. Gen. Microbiol. 1, 314-326 (1947)

Gardner, A.D.: Morphological effects of penicillin on bacteria. Nature 146, 837-838 (1940)

Gros, F., Macheboeuf, M.: Recherches biochemiques sur le mode d'action de la pénicilline sur un bactérie: *Clostridium sporogenes*. Ann. Inst. Pasteur 74, 368-385 (1948)

Hahn, F.E., Ciak, J.: Penicillin-induced lysis of *Escherichia coli*. Science 125, 119-120 (1957)

Hobby, G.L., Meyer, K., Chaffee, E.: Observations on the mechanism of action of penicillin. Proc. Soc. Exp. Biol. Med. 50, 281-285 (1942)

Hotchkiss, R.D.: The abnormal course of bacterial protein synthesis in the presence of penicillin. J. Exp. Med. 91, 351-364 (1950)
Hunter, T.H., Baker, K.T.: The action of penicillin on *Bacillus subtilis* growing in the absence of amino acids. Science 110, 423-425 (1949)
Jawetz, E., Gunnison, J.B., Speck, R.S., Coleman, V.R.: Studies on antibiotic synergism and antagonism. Arch. Int. Med. 87, 349-359 (1951)
Johnstone, K.I., Crofts, J.E., Evans, D.G.: Single cell culture of Cl. welchii type A morphologically changed by penicillin. Brit. J. Exp. Pathol. 31, 562-565 (1950)
Kochen, M. (ed.): The Growth of Knowledge. New York: Wiley 1967
Krampitz, L.O., Werkman, C.H.: On the mode of action of penicillin. Arch. Biochem. 12, 57-67 (1947)
Lamanna, C., Shapiro, I.M.: Sulfanilamide bacteriostasis in presence of mercuric chloride and p-aminobenzoic acid. J. Bacteriol. 45, 385-394 (1943)
Lederberg, J.: Bacterial protoplasts induced by penicillin. Proc. Natl. Acad. Sci. USA 42, 574-577 (1956)
Lederberg, J.: Mechanism of action of penicillin. J. Bacteriol. 73, 144 (1957)
Lederberg, J., St. Clair, J.: Protoplasts and L-type growth of *Escherichia coli*. J. Bacteriol. 75, 143-160 (1958)
Liebermeister, K., Kellenberger, E.: Studien zur L-Form der Bakterien. I. Die Umwandlung der bazillaren in die globulare Zellform bei Proteus unter Einfluss von Penicillin. Z. Naturforsch. 11b, 200-206 (1956)
Mitchell, P.: Penicillin and the logic of chemotherapy. Giorn. Microbiol. 2, 440-460 (1956)
Mitchell, P., Moyle, J.: Relationships between cell growth, surface properties and nucleic acid production in normal and penicillin-treated *Micrococcus pyogenes*. J. Gen. Microbiol. 5, 421-438 (1951)
Nature 156, 766-767 (1945): Chemistry of Penicillin
Park, J.T.: Isolation of three labile phosphate compounds containing uracil from penicillin-treated *Staphylococcus aureus* cells. Fed. Proc. 9, 213 (1950)
Park, J.T.: Uridine-5'-pyrophosphate derivatives. I. Isolation from *Staphylococcus aureus*. J. Biol. Chem. 194, 877-884 (1952a)
Park, J.T.: Uridine-5'-pyrophosphate derivatives. II. A structure common to three derivatives. J. Biol. Chem. 194, 885-895 (1952b)
Park, J.T.: Uridine-5'-pyrophosphate derivatives. III. Amino acid-containing derivatives. J. Biol. Chem. 194, 897-904 (1952c)
Park, J.T., Johnson, M.J.: Accumulation of labile phosphate in *Staphylococcus aureus* grown in the presence of penicillin. J. Biol. Chem. 179, 585-592 (1949)
Park, J.T., Strominger, J.L.: Mode of action of penicillin. Biochemical basis for the mechanism of action of penicillin and for its selective toxicity. Science 125, 99-101 (1957)
Pratt, R., Dufrenoy, J.: Antibiotics. Philadelphia: Lippincott 1949
Prestidge, L.S., Pardee, A.B.: Induction of bacterial lysis by penicillin. J. Bacteriol. 74, 48-59 (1957)
Reid, R.D.: Some properties of a bacterial-inhibitory substance produced by a mold. J. Bacteriol. 29, 215-221 (1935)
Rowley, D., Miller, J., Rowlands, S., Lester-Smith, E.: Studies with radioactive penicillin. Nature 161, 1009-1010 (1948)
Science 102, 627-629 (1945): Chemistry of Penicillin
Schepartz, S.A., Johnson, M.J.: The nature of the binding of penicillin to bacterial cells. J. Bacteriol. 71, 84-90 (1956)
Stent, G.S.: Prematurity and uniqueness in scientific discovery. Sci. Am. 227 (6), 84 (1972)

Strange, R.E., Kent, L.H.: The isolation, characterization and chemical synthesis of muramic acid. Biochem. J. 71, 333-339 (1959)

Umbreit, W.W., Burris, R.H., Stauffer, J.F.: Manometric techniques and relared methods for the study of tissue metabolism. Minneapolis: Burges 1945

Wilson, D.: In Search of Penicillin, pp. 111-115. New York: Knopf 1976

END

DATE
FILMED

3-84

DTIC