Progress in Molecular and Subcellular Biology

8

With Contributions by
J. R. Bamburg, T. L. German, F. E. Hahn
G. Knudson, R. F. Marsh, J.J. O'Neill

Edited by F. E. Hahn, D. J. Kopecko
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Professor Fred E. Hahn, Ph. D.
Division of Communicable Diseases and Immunology
Walter Reed Army Institute of Research
Washington, D.C. 20307, USA

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Contributors

BAMBURG, J.R., Chairman, Department of Biochemistry, Colorado State University, Ft. Collins, CO 80523, USA

GERMAN, T.L., Department of Veterinary Science, 1655 Linden Drive, University of Wisconsin, Madison, WI 53706, USA

HAHN, F.E., Walter Reed Army Institute of Research, Division of Communicable Diseases and Immunology, Washington, D.C. 20307, USA

KNUDSON, G., U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Frederick, MD 21701, USA

MARSH, R.F., Department of Veterinary Science, 1655 Linden Drive, University of Wisconsin, Madison, WI 53706, USA

O'NEILL, J.J., Chairman, Pharmacology Department, Temple University, 3420 N. Broad Street, Philadelphia, PA 19140, USA

Contents

F.E. HAHN: Penicillin Until 1957 (With 11 Figures)

A. Introduction 1
B. The Discovery of Penicillin 2
C. Penicillin as a Chemotherapeutic Drug 4
D. Early Studies on Mode of Action 5
E. Paradoxical Inhibitory Zone Phenomena 6
F. Morphological Changes in Bacteria 8
G. Bacterial Lysis by Penicillin 10
H. Interference of Penicillin with Nucleic Acid and Protein Synthesis? 12
I. Park's Nucleotides 14
J. Penicillin Binding by Bacteria 16
References 19

G.B. KNUDSON: The Role of Inducible DNA Repair in W-Reactivation and Related Phenomena

A. Introduction 22
B. Enzymatic Repair Systems in Bacteria and Their Viruses 22
C. W-Reactivation and W-Mutagenesis 24
D. Direct and Indirect Prophage Induction 30
E. Plasmid Mediated UV-Protection and Mutagenesis 31
F. Inducible "SOS"-Repair Functions 31
References 33

J.R. BAMBURG: Biological and Biochemical Actions of Trichothecene Mycotoxins (With 9 Figures)

A. Introduction 41
B. Trichothecene Structure and Genesis 43
C. Detection of Trichothecenes 55
D. Trichothecene Production 58
E. Human and Animal Mycotoxicoses in which Trichothecenes are the Suspected Causative Agents 62
F. Biological Activity of the Trichothecenes 69
G. Biochemical Studies on the Action of Trichothecenes 72
H. Physiological Mechanisms of Trichothecene Toxicity 76
I. Metabolism of Trichothecenes 88
J. Trichothecenes - Pharmaceuticals or Chemical Weapons ... 92
References 94

T.L. GERMAN and R.F. MARSH: The Scrapie Agent: A Unique Self-Replicating Pathogen (With 4 Figures)

A. Introduction 111
B. Biology 111
C. Biochemical Characterization 113
D. Theoretical Considerations for a Self-Regulated Protein Pathogen 114
E. Future Directions 118
References 119

J.J. O'NEILL: Non-Cholinesterase Effects of Anticholinesterases (With 12 Figures)

A. Background 122
B. Neuropeptides 127
C. Conclusion 138
References 140

Subject Index 145

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.*

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

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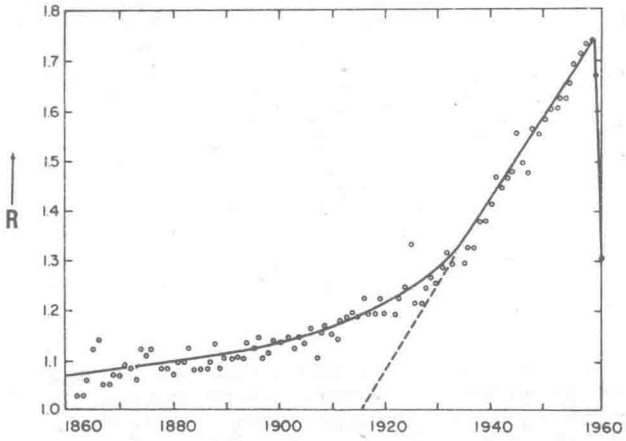

Fig. 1. Ratios of the number of 1961 citations to the number of papers published in each of the years 1860 through 1960 (De Solla Price in Kochen, 1967)

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.

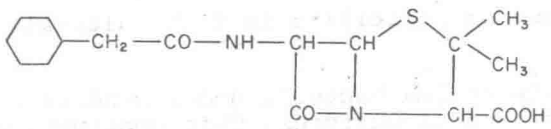

Fig. 2. Structure of Penicillin G

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

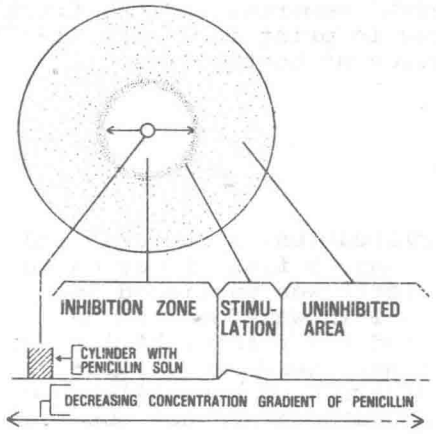

Fig. 3. Diagrammatic representation of a penicillin assay plate, showing ring of enhanced growth of *Staphylococcus aureus* on uniformly seeded culture plates supplied with a cyclinder of penicilling solution: surface view above, sectional view below. (Pratt and Dufrenoy 1949)

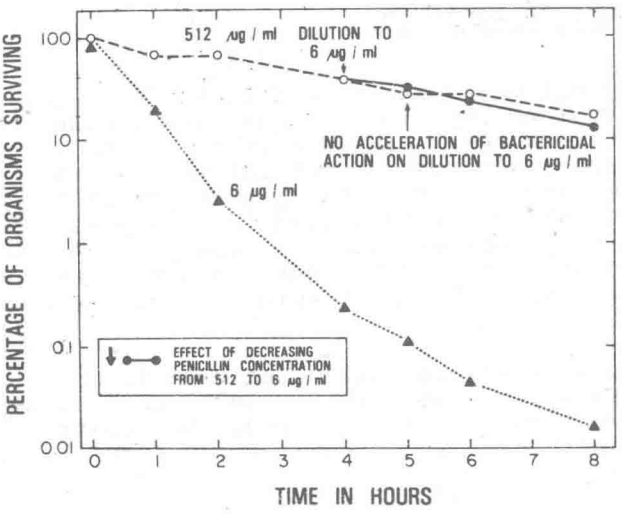

Fig. 4. The persistent effect of brief exposure to high concentrations of penicillin on the death rate of *Streptococcus faecalis* (Eagle 1951)

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.

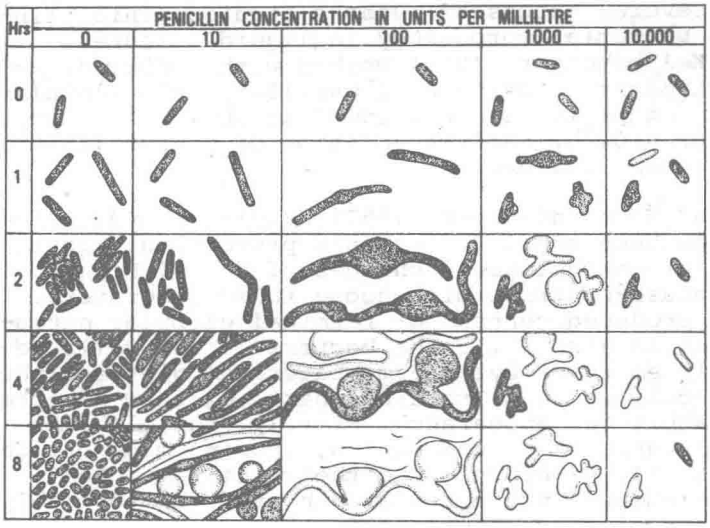

Fig. 5. Morphological effects of penicillin on growing *Escherichia coli* (Duguid 1946)

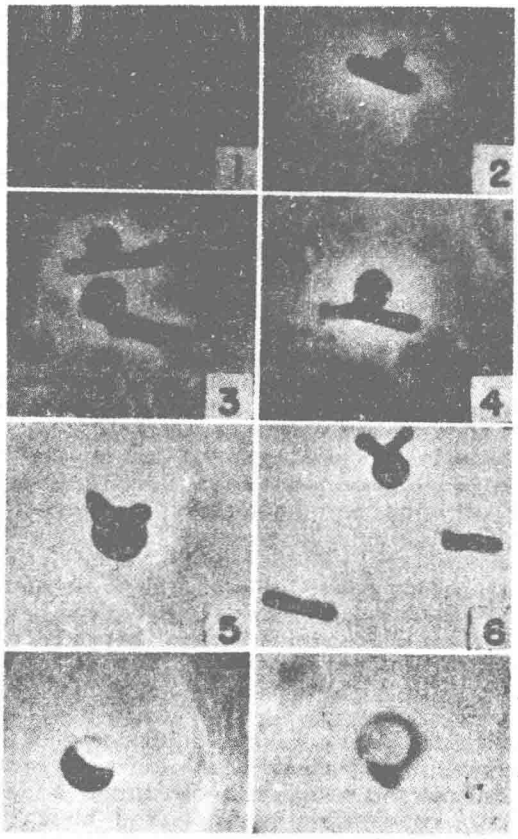

Fig. 6. Morphological effects of penicillin on *Escherichia coli*, growing in liquid medium with 0.48 M sucrose for osmotic protection (Hahn and Ciak 1957)

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* culture 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

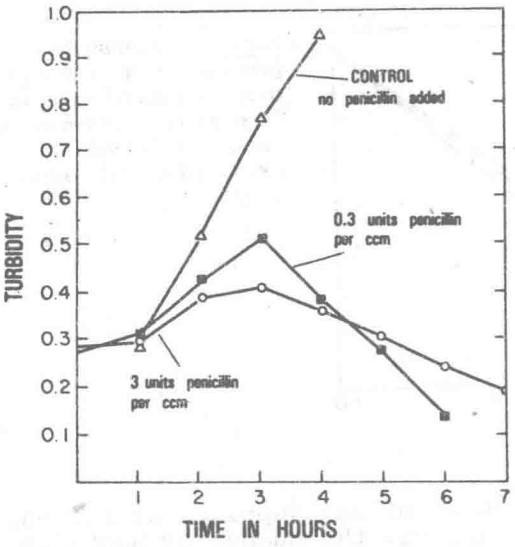

Fig. 7. Penicillin-induced lysis of *Staphylococcus*, growing in liquid medium (Chain and Duthi 1945)

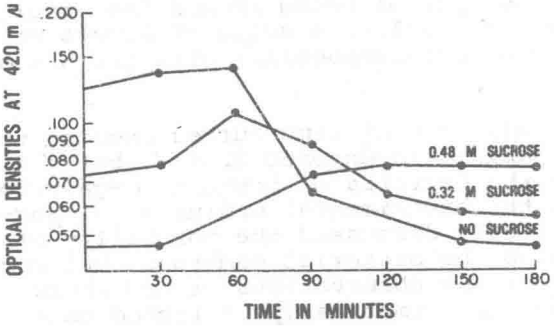

Fig. 8. Penicillin-induced lysis of *Escherichia coli* growing in liquid media with and without sucrose for osmotic protection (Hahn and Ciak 1957)

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-