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Mode of action of antibiotics

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Thesis

MODE OF ACTION OF ANTIBIOTICS

by

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I wish to express my sincerest gratitude to Dr. Genevieve Young of the Department of Biology, whose guidance and patience have made this thesis possible.
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INTRODUCTION

"La vie empêche la vie"—Pasteur

Today an antibiotic (anti, against; bios, life) may be defined as an organic substance produced by a living organism which inhibits the growth of or kills some other living organism. The term originated in 1877 when Pasteur and Joubert noted that air-borne bacteria inhibited the growth of the anthrax bacillus and said that "among the lower beings, even more than among the higher animals and plant species, life destroys life".

Most of the work in this field has concerned itself with the discovery of large numbers of antibiotics, and the determination of their anti-bacterial spectra and clinical activities. Therefore, since very little experimental work has been done on the mechanisms by which antibiotics exert their effects or on the basis for bacterial resistance, I felt that a progress report of the mode of action of some of the more important antibiotics, such as penicillin and streptomycin, would be appropriate at this time.

According to Hotchkiss (1948), most of the experimental approaches in the field of antimicrobial activity represent direct attempts to observe one or more of the following events: (a) accumulation of a toxic concentration of the antibiotic in the affected cell, either by simple diffusion, adsorption, or active absorption; (b) interaction of the
agent at this concentration with some structural element, or with some metabolic constituent of the cell; (c) interference by this interaction with a normal cellular function; and (d) the gradual or immediate alteration in the biochemical growth processes of the cell as a consequence of this interference.

Since no antibiotic activity is fully explained until something can be said about all of these four aspects, I shall attempt, in my review of the literature, to fulfill the criteria mentioned above. In this way, the entire subject will be brought into focus as a single entity for those research investigators interested in antibiotic activity.

For the purpose of discussion, the mechanism of action of antibiotics will be considered with reference to one or more of the following levels of organization of the bacterial cell: (a) the active group or groups in the antibiotic which may be effective against the bacterial cell; (b) the effect of the antibiotic on the morphology (cytology) of the intact cell; (c) the action of the agent on bacterial metabolism; (d) environmental factors influencing antibiotic activity; (e) a study of the origin of bacterial resistance; and (f) combined activity between key antibiotics and other chemotherapeutic agents.

As a guide in reading the thesis, I have included Chart 1 taken from Modern Pharmacy (1951), which shows the range of activity of three antibiotics - penicillin, streptomycin,
CHART 1

The Effective Range of Antibiotics
and chloromycetin - extending from the larger micro-organisms, the yeasts, to the smallest of the viruses.
PENICILLIN

Background
According to Ratcliff (1945), the development of penicillin may be summarized in four major steps: (1) Alexander Fleming's (1929) initial report of the discovery of a contaminating mold, Penicillium notatum, among culture plates of staphylococcal variants; (2) the ten-year period (1930-1940) during which English investigators, such as Chain, Florey et al. (1940), studied the chemotherapeutic and clinical uses of the drug; (3) the contribution of American industry in cooperation with the Office of Scientific Research and Development (OSRD) in undertaking full-scale production of penicillin; and finally (4) the magnificently organized clinical study of this antibiotic by the National Research Council's Committee on Chemotherapeutic and Other Agents, under the direction of Chester Keefer (1943).

Structure
(Chemical Formula)
Although Fleming (1929) used "penicillin" to describe the total antibacterial activity produced by a particular mold, the term has come to mean a group of chemical compounds, biosynthesized by several species of molds, notably penicillia belonging to the notatum-chrysogenum group.

Winsten and Spark (1947) observed that Penicillium chrysogenum Q-176, when grown on a corn steep liquor-lactose
medium, could produce no less than eight penicillins, three of which, S-1, S-2, and S-3, related to penicillin X, have not as yet been characterized. Therefore, as indicated in Table 1, taken in part from Rake and Richardson (1948) and Pratt and Dufrenoy (1948b), only five penicillins are commonly encountered, each possessing the same fundamental chemical structure, but differing in variations of R.

Complete structural formulae for the penicillin molecule were first proposed by Abraham et al. (1944), who showed that penicillin has one or the other of the general formulae shown below:

(1)

\[
\text{NH-CH} \quad \text{CH} \quad \text{C\text{(CH}_3\text{)}_2} \\
\quad \text{R-CH} \quad \text{N-CH}_2\text{COOH}
\]

Beta-Lactam Formula

(2)

\[
\text{NH-CH} \quad \text{CH} \quad \text{C\text{(CH}_3\text{)}_2} \\
\quad \text{R-CH} \quad \text{N-CH}_2\text{COOH}
\]

Azlactone (Oxazolone) Formula

A third formula for penicillin, involving a tricyclic structure, was proposed by Bentley et al. (1944) as follows:

(3)

\[
\text{O-CH} \quad \text{C(\text{CH}_3\text{)}_2} \\
\quad \text{R-CH}_2\text{COOH}
\]

Tricyclic Formula
<table>
<thead>
<tr>
<th>Name</th>
<th>Synonym</th>
<th>Source</th>
<th>R Group</th>
<th>Carboxyl Residue</th>
<th>Acetamide Linkage</th>
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<tr>
<td>F</td>
<td>I</td>
<td><em>P. chrysogenum-notatum</em></td>
<td>3-hexenoic</td>
<td>2-pentenyl</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>II</td>
<td>&quot;</td>
<td>phenylacetic</td>
<td>benzyl</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>III</td>
<td>&quot;</td>
<td>p-hydroxy-phenyl-acetic</td>
<td>p-hydroxy-benzyl</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>IV</td>
<td>&quot;</td>
<td>octanoic</td>
<td>n-heptyl</td>
<td></td>
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<tr>
<td>Dihydro F</td>
<td>Gigantic acid</td>
<td><em>Aspergillus giganteus</em></td>
<td>caproic</td>
<td>n-amyl</td>
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Although this latter structure offered a ready explanation for the formation of penicillic acid from penicillin in dilute acid, it was not acceptable to other workers because it did not give a convincing illustration of the non-basic character of the nitrogen atoms in the penicillin molecule.

It is interesting to note that Pratt and Dufrenoy (1948a) represent the fragments that may be obtained through hydrolytic cleavage of penicillin as follows:

\[
\begin{align*}
\text{R Residue} & \quad \text{Cysteiny}l \quad \text{Residue} \quad \text{Dimethylserine} \quad \text{Residue} \\
& \quad | \\
& \quad \text{H} \quad \text{H} \quad \text{H} \\
& \quad | \\
& \quad \text{H} \quad \text{H} \\
& \quad | \\
& \quad \text{H}
\end{align*}
\]

However, Behrens (1949) believes that the S atom, at cleavage, remains as part of the beta-dimethylcysteinylo residue. Thus, penicillin may be thought of as a ring condensation of two amino acids - alanine and beta-dimethylcysteine:

\[
\begin{align*}
\text{R Residue} & \quad \text{Alanyl} \quad \text{Residue} \quad \beta\text{-dimethylcysteinylo Residue} \\
& \quad | \\
& \quad \text{H} \quad \text{H} \quad \text{H} \\
& \quad | \\
& \quad \text{H} \quad \text{H} \\
& \quad | \\
& \quad \text{H}
\end{align*}
\]

Since Hotchkiss (1948) found little indication of dif-
ferences in the mode of action of the various penicillins, F, G, K, X, etc., the generic term "penicillin" will be best construed as applying to any of the types, unless a particular kind is specified.

Effect on Morphology (Cytology) of Bacterial Cell

Gardner (1940) was the first investigator to point out the striking changes in the shape of bacteria after exposure to varying concentrations of penicillin. *Clostridium welchii*, *Eberthella typhosa*, *Vibrio cholerae*, and even *Escherichia coli* all showed elongation and swelling of their cells. Streptococci also showed concatenation of the cells, while staphylococci became spherically enlarged. He interpreted these results by postulating that growth took place in the bacteria, but there was a failure of fission. Thus, normal division did not follow and many cells then underwent autolysis.

Smith and Hay (1942) noted a marked increase in the size of actively growing staphylococci exposed to inhibitory concentrations of penicillin. He found that lysis then occurred with the formation of small masses of granular material - evidently the protoplasmic substances released by the breakdown of the cell wall. Since fully grown suspensions of these organisms did not show these changes, the authors concluded that penicillin either acted directly on the cellular wall or prevented the assimilation of certain
growth factors necessary for the actual fission of the growing cell.

In vivo studies by Miller et al. (1944) on the effect of penicillin on gonococcal urethritis revealed that, as early as two hours after intramuscular injection of the drug, a large proportion of the gonococci were swollen, irregular in shape, unequally stained, and surrounded by clear zones. According to Miller and Foster (1944), meningococci were similarly affected in vitro by inhibitory concentrations of penicillin. Distorted and enlarged forms of meningococci suggested inability of the cells to complete the normal process of fission.

Levaditi and Vaisman (1945), by the argyrophilic staining method, studied the action of penicillin on the morphology of Staphylococcus aureus, E. coli, Bacilli paratyphosa A and B, and Spirochaeta duttoni. At eight hours, the bacterial cells swelled and emptied their contents; this resulted in the suppression of their ability to stain with silver dyes. After the third or fourth day, the cells were transformed into stromas, which persisted for a long time in the penicillin culture. The loss of the ability to stain with silver nitrate was attributed either to the fact that the protein-carbohydrate-lipoid portion of the membrane could not fixate the AgNO₃, or the fixated nitrate was no longer reducible.
In a study of the effect of penicillin on spores and vegetative cells of *Bacillus anthracis* and *Bacillus subtilis*, Gardner (1945) observed that strong concentrations of penicillin (50 to 100 units per ml.) caused the spores that were grown on an agar medium to lose their high refractivity and become empty ghosts. Weaker concentrations (0.1 to 1.0 unit per ml.), however, allowed the germination of the spores to occur; this was followed by swelling until they became spherical coccoids which burst. As a result, the great majority of the spores were killed. In non-nutrient media, the spores were little affected, even by strong concentrations of penicillin. Thus, the conclusion was reached that the penicillin acted on feeding bacteria immediately when they began to feed. Thus, the activity of penicillin on sensitive bacteria had little or no connection with multiplication or division.

Thomas and Levine (1945) observed that changes in cultural characters and bizarre involution forms were produced by gram-negative bacilli of the intestinal tract when grown in liquid and solid media containing inhibitory, but not completely bacteriostatic, concentrations of penicillin. For example, growth in liquid media, instead of being uniformly distributed throughout the liquid, was granular and restricted to the surface in some cultures and to the bottom in others. On nutrient agar, as the penicillin concentration was increased, the butyrous consistency, so
characteristic of culture of the intestinal group, was supplanted by a rubbery adherent growth. The involution forms varied from long, twisting filaments ten to several hundred times the normal cells in the lower inhibitory concentrations to such bizarre forms as cells resembling deformed integration signs, Pasteur flasks, swelled fusiform bacilli, large globular cells, and pleomorphic forms when growth was present in higher concentrations.

Alture-Werber et al. (1945), in studying morphological variants of *E. coli*, found that distinct changes in structure from the usual short rod appearance took place at a penicillin concentration of 100 Oxford units per ml. At this point, the majority of the cells took the form of unsegmented filaments many times longer than the average normal bacillus. In 150 Oxford units per ml., bipolar rounded cells, appearing like budding fungi, were present.

Kojima and Heimbrock (1946) observed peculiar, large, actively motile, gram-negative, elongated organisms with a central globular or oval swelling in the urine of a patient receiving penicillin. These bodies resembled budding fungi similar to those reported by Alture-Werber et al. (1945). It was suggested that these forms were involution forms of *E. coli* since this organism always grew out when the urine sediment was cultured upon ordinary media. Extreme morphological variants of *Bacillus aerogenes* were produced by
Fennel (1946), using inhibitory concentrations of penicillin. These organisms were non-motile and, as above, resembled some strange sporulating form of fungus.

Fisher (1946) studied the effect of penicillin on the morphology of the staphylococcus, streptococcus, and pneumococcus. He noted that the in vitro action of penicillin on staphylococci caused enlargement of the bacterial cells followed by lysis. This effect was apparently due to interference with the normal stimulation to cell division and multiplication rather than to complete inhibition of growth. This same effect was apparent to a lesser degree on cultures of beta hemolytic streptococci and pneumococci. Since, in the case of the pneumococcus, the capsule remained intact for some time after the cell body was destroyed, this investigator believed that the penicillin either passes through the capsule by diffusion and then destroys the organism, or is actively absorbed by the growing pneumococcus and passes through its capsule during the process of bacterial metabolism. However, when he observed that penicillin only acted on dividing bacteria - as shown by its failure to produce morphological or cultural changes in very old cultures or at icebox temperatures - the latter theory seemed more likely to be correct. Thus, the conclusion was presented that penicillin does not merely have a physical or chemical effect but influences some phase of the organism's metabolic and reproductive activity.
Frazier and Frieden (1946) studied the effect of penicillin on the morphology of Treponema pallidum. They found that there was an increase in the number of long forms of the spirochete which might be an indication of excessive growth and delayed cell division. In addition, the small zone of thickening at the point of articulation indicated to these writers that the organism may possibly divide by binary fission.

Shanahan et al. (1947), by means of the type B, RCA electron microscope, showed structural changes in E. coli as a result of exposure to penicillin. At lower concentrations, there was little change in morphology, but as the unitage was increased, more elongated and swollen cells appeared, many of them remaining only partially divided. The appearance of very large, fusiform bodies was noted especially at 150 and 200 units per ml. In addition an intense granulation of the fusiform and rod forms was revealed by electron microscopy.

Shanahan and Tanner (1948), in further studies on the morphology of E. coli, exposed twenty-four strains to various concentrations of penicillin in nutrient broth, in MacConkey's agar plates, and on slide cultures. By light microscope studies, they observed that all strains responded under one condition or another, by producing morphological variants. Among the forms seen were elongated swollen rods, filamentous rods, filamentous rods with fusiform structures...
usually near the middle of the cell, terminal or lateral bulb structures, spindle forms, and occasionally a large round form.

Hughes et al. (1949) used the phase-contrast microscope to observe changes in morphology in such bacteria as *Proteus vulgaris*, *E. typhosa*, *E. coli*, and *V. cholerae* when they were exposed to sublethal doses of penicillin. The changes seen were (1) enormous elongation and thickening of the rods, (2) production of single or multiple swellings on the rods, and (3) branching of the rods.

Pratt and Dufrency (1947a), by treatment with vital dyes, studied the cytological changes that occurred in living cells of *S. aureus* exposed to bacteriostatic or bactericidal concentrations of penicillin. For cytological examination, *S. aureus* test organisms were removed with a platinum loop from different parts of test plates and were suspended in a drop of vital dye such as neutral red. The cells which were collected from the uninhibited background outside the zones of inhibition (normal cells) were compared with those taken from different locations within zones surrounding cylinders containing solutions with concentrations of penicillin ranging from 0.5 to 8 Oxford units per ml.

Figure 1-A represents diagrammatically the behavior of a normal, living cell of *S. aureus* exposed to a solution of neutral red. It may be seen that the dye accumulates in the vacuole of the cells (solid black), and that cell division
FIGURE 1

DIAGRAMMATIC REPRESENTATION OF STAPHYLOCOCCUS aureus
STAINED VITALLY WITH NEUTRAL RED

A

B
occurs without hindrance. Figure 1-B is a similar representation of the behavior of cells subjected to a solution of neutral red following exposure to a bacteriostatic concentration of penicillin. Successive diagrams from left to right show that the cell swells; material staining with neutral red diffuses from the vacuole to the periphery; the cell fails to divide and eventually swells into an empty shell, which shows diffuse bipolar peripheral staining.

These changes in structure were followed by failure of the vacuoles not only to absorb materials from the external environment, but to retain material enclosed within them. It may be noted that this condition occurred concomitantly with the disorganization of the cellular nucleoprotein and the liberation of lipids and fatty acids.

Similarly, Pratt and Dufrenoy (1948a), by microscopic examination of cells taken from several cultures of S. aureus and B. subtilis grown in broth containing penicillin with or without 1 mg. of CoCl₂·6H₂O per liter, showed that the same morphological and cytological changes occurred in the cobalt series as in the controls, but at a lower concentration. There was a tendency to change from the normal colonial habit of growth, i.e. normal three-dimensional colonies, to the formation of "streptococcus-like" chains of swollen cells which may become arranged in plates and finally separate to form "diplococcus-like" structures that show bipolar staining with vital dyes instead of the normal tendency to
accumulate such dyes in the central vacuolar portion. Coincident with these phenomena, the cells released their ribonucleic materials into the medium, and lost their positive reaction to the gram stain.

These same men showed a similar sequence of events from seeded assay plates preincubated for three hours without penicillin and then reincubated for a second period of three hours during which penicillin was diffusing from the cylinders. Figure 2 represents a portion of an inhibition zone and adjacent regions of the assay plate. It may be observed that the original micro colonies that form during the preincubation period continue, during the second incubation period, to proliferate three-dimensionally in the background where they do not fall under the influence of enhancing or depressing concentrations of penicillin. In addition, they are able to absorb or concentrate neutral red in the vacuolar solution (black in diagram). However, with enhancing concentrations of penicillin, the original colonies no longer proliferate three-dimensionally, mostly within the agar, but, instead, the newly-formed cells spread in one plane, forming "streptococcus-like" chains that may lie close together in a plate-like arrangement on the surface of the agar. These changes are probably correlated with the release of cellular constituents from the swelling organisms and can be shown by the use of vital
FIGURE 2

SCHEMATIZED DIAGRAMMATIC REPRESENTATION OF MORPHOLOGICAL AND STAINING CHARACTERISTICS OF CELLS OF STAPHYLOCOCCUS aureus IN DIFFERENT REGIONS OF A PENICILLIN ASSAY PLATE INCUBATED AT 37°C

INHIBITION ZONE
- PENICYLINDER
- DIFFUSION OF PENICILLIN AND PHOSPHATE

OUTWARD DIFFUSION OF LYSIS PRODUCTS FROM CELLS IN THIS AREA PROMOTES RAPID GROWTH OF ADJACENT CELLS, THUS RENDERING THEM MORE SUSCEPTIBLE TO PENICILLIN

STIMULATION

UNINHIBITED BACKGROUND

CELLS IN DIPLOCOCCUS FORM - STAIN BIPOLAR

CELLS SWOLLEN

NORMAL COLONIES STAIN IN CENTRAL VACUOLE OF EACH CELL - COLONIES THREE DIMENSIONAL

CHAINS, "DIPLO" FORMS STAIN PERIPHERAL BIPOLAR, OR NONE

CHAINS Y PLATES

ORIGINAL COLONIES

19
dyes, since the staining, when it occurs, is peripheral. Then, under slightly higher, though still sub-bacteriostatic concentrations, the cells become more loosely associated and finally resolve into "diplococcus-like" structures that show bipolar staining. Furthermore, it will be seen that the profile of the agar structure indicates a slightly raised level in the zone of enhanced growth wherein the material diffusing from the lysed organisms in the inhibition zone is actively metabolized. This tendency of the proliferating chains and plates of cells in the zone of enhanced growth to crowd the surface may reflect an "oxygen hunger" induced by such cells, which is probably the result of two factors: (1) Direct stimulation by an appropriately low concentration of penicillin. (2) An accelerated rate of metabolism induced by the availability in this region of "growth factors" released from the lysed cells in the inhibition zones.

Other work by Dufreney and Pratt (1947b) revealed that cells of *S. aureus* lost their gram-positive staining reaction when exposed to bacteriostatic concentrations. Thus, within the inhibition zones on standard sixteen-hour agar penicillin assay plates, where no positive reaction for sulfhydryl groups can be obtained, cells of *S. aureus* are no longer gram-positive. It is notable that cells affected by penicillin, as they are in the process of division, swell into "diplococcus-like" units, and the gram-positive material appears as inclusions in two sharply defined regions, one
towards each pole. Thus, each of these gram-positive regions is homologous to the portions of the cells previously described as staining vitally with neutral red under a comparable stage of inhibition by bacteriostatic concentrations of penicillin.

An interesting example of the action of penicillin in cellular formation was the observation by Cornman (1944) concerning the effect of penicillin (Squibb's sodium salt) on normal and malignant (sarcoma) cells of the rat and mouse. In roller tube cultures, sarcoma cells were grown with normal fibroblasts derived from explants of muscle from the same strain of tumor host. The addition of penicillin revealed that two or three times as much penicillin was required to damage normal cells as was required to cause damage to malignant cells. Furthermore, this experimenter was unable to produce tumors in rats by implantation of the malignant tissue that had been exposed to penicillin, whereas the untreated control tumor cultures regularly produced tumors.

Lewis (1944) compared the effect of a more highly purified penicillin (Merck's sodium salt) with that of the yellow salt that was used by Cornman (1944). She was unable to demonstrate any retardation of the growths of grafts of sarcoma in mice by the highly purified colorless sodium salt of penicillin in contrast to the inhibitory effect of the yellow (unpurified) sodium salt. Thus, it would appear that the factor present in the less purified salt, which damaged
the sarcoma cells, was lost in the highly purified product.

**Effect on Bacterial Metabolism**

**Effect on Amino Acid and Protein Metabolism**

Since Taylor (1947) observed that the ability to concentrate amino acids, such as glutamic acid, was a property of gram-positive organisms, Gale and Taylor (1947) decided to investigate the effect of penicillin on glutamic acid assimilation. Table 2 shows the amount of glutamic acid absorbed by growing cells of *S. aureus* which were harvested at intervals after the addition of various concentrations of penicillin to the cultures. It may be observed that cells taken from cultures to which no penicillin had been added accumulated 560-700 μl. glutamic acid per 100 mg. However, within thirty minutes after the addition of penicillin (10 units/ml.) to the cultures, the assimilatory power of the cells had fallen to 1/4% of that of the control. Within one hour, the concentration had fallen further to 1/4% of that of the control and, finally, after one and one-half hours, the absorption of glutamic acid was no longer possible. Since penicillin was found to have no effect upon the assimilation of glutamic acid in resting cells, these investigators postulated that penicillin either combines with or produces a reorganization of the cell wall of growing cells such that the assimilatory mechanism is blocked.
### TABLE 2

**EFFECT OF THE PRESENCE OF PENICILLIN DURING GROWTH ON THE ASSIMILATION OF GLUTAMIC ACID BY STAPHYLOCOCCUS aureus**

<table>
<thead>
<tr>
<th>Penicillin concentration (units per ml. of medium)</th>
<th>Glutamic acid assimilated (µg./100 mg. cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time of harvesting after penicillin addition</td>
</tr>
<tr>
<td></td>
<td>30 min.</td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>5.0</td>
<td>-</td>
</tr>
<tr>
<td>10.0</td>
<td>82</td>
</tr>
</tbody>
</table>
Penicillin, however, did not interfere with the internal metabolism of glutamic acid. This was demonstrated by these same men using the principle that, if the amount of glutamic acid which accumulated inside the cell during assimilation was compared to the amount which was removed from the external environment, there would be on balance a 'disappearance' of the amino acid. The results in Table 3 suggest that the internal metabolism of glutamic acid continued normally in the penicillin-treated cells, but, since the passage of glutamic acid across the cell wall was blocked, this metabolic process took place at the expense of the internal glutamic acid already present. On the other hand, in the normal cells the glutamic acid metabolized internally is balanced by assimilation forces from the external environment and by an increase in the internal level to maintain equilibrium with the external concentration.

Gale (1947a) noted that, since gram-negative bacteria, in general, are able to synthesize glutamic acid and consequently do not need to assimilate the free amino acid, these organisms would not be expected to be sensitive to the action of a substance, such as penicillin, whose only function was to prevent such assimilation. However, it has been demonstrated that some gram-negative bacteria, especially meningococci and gonococci, are sensitive to the action of penicillin.

In this connection, experiments were done by Pratt and
### TABLE 3

**EFFECT OF PENICILLIN ON GLUTAMIC ACID METABOLISM**

**OF STAPHYLOCOCCUS aureus**

<table>
<thead>
<tr>
<th></th>
<th>External environment</th>
<th>Internal environment</th>
<th>Change in external environment (μl. glutamic acid)</th>
<th>Change in internal environment</th>
<th>Glutamic acid metabolized</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal cells:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>1586</td>
<td>690</td>
<td>-831</td>
<td>±370</td>
<td>461</td>
</tr>
<tr>
<td>Final</td>
<td>755</td>
<td>1060</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Penicillin-treated cells:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>1646</td>
<td>579</td>
<td>-106</td>
<td>-263</td>
<td>363</td>
</tr>
<tr>
<td>Final</td>
<td>1540</td>
<td>315</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Dufrenoy (1947b) on physical and chemical changes that occur in different parts of penicillin assay plates seeded with gram-positive (S. aureus and B. subtilis) and gram-negative (E. coli and P. vulgaris) organisms. Their results showed that, in general, essentially the same pattern appeared on penicillin assay plates subjected to a given reagent, irrespective of the test organism, provided that the proper concentration of penicillin was used and the proper balance of biological factors, represented by the growth of the test organism, and of physical factors, represented by the diffusion of penicillin, was achieved. For example, on plates seeded with S. aureus, a solution of one unit of penicillin per ml. produced inhibition zones approximately 21 mm. in diameter, whereas a solution containing approximately 100 units of penicillin per ml. was required to produce a zone of the same diameter on plates seeded with P. vulgaris. Therefore, they concluded that penicillin affects gram-positive and gram-negative organisms through the same chemical systems; however, the threshold concentration at which the effects of penicillin become manifest is many times greater on plates seeded with gram-negative organisms than in those seeded with gram-positive ones.

Dufrenoy and Pratt (1947b) believe that the observation that penicillin inhibits the passage of glutamic acid across the cell wall is extremely significant, since this amino acid is a component of glutathione, the activity of the -SH group
of which is known to depend in large measure on the neighboring NH groups. Therefore, it was desirable to study the distribution of amino acids in various regions of penicillin assay plates after different periods of exposure of cells of \textit{S. aureus} to penicillin. The Sakaguchi test for the guanidine groups of arginine, the alloxan test for $\alpha$-amino acids, and Millon's test for tyrosine all indicated the absence of amino acids in the zone of inhibition, and their presence in the zone of enhanced growth.

Furthermore, Pratt and Dufrenoy (1948a) have commented on the similarity in structure between glutathione, a tripeptide, and penicillin. The various penicillins may be written as stereochemical analogues of glutathione with glycine replaced by dimethyl serine, and the glutamyl fraction replaced by an R residue as follows:

\[
\begin{align*}
\text{Glutamic Acid} & \quad \text{Cysteine} & \quad \text{Glycine} \\
\text{Glutathione} & \quad \text{Penicillin}
\end{align*}
\]
Because of this similarity in structure, Fischer (1947) thinks of the possibility of penicillin competing with glutathione for enzymatic or other mechanisms important for microbial reproduction.

Dufreney and Pratt (1947a) have reported on some cytochemical mechanisms of penicillin action, based upon the addition of suitable reagents and observations of the reactions in areas on agar plates which show bacterial growth as compared with zones containing penicillin-inhibited micro-organisms (S. aureus and B. subtilis). The techniques employed were intended to yield information about oxidation-reduction levels in different parts of the plates, and were based on the observation of Genevois and Cayrol (1939) that, in the normal aerobic respiration of the test organism, a balance exists between the rate of dehydrogenation of -SH groups effective in hydrogen transfer and the rate of restitution of such groups. Thus, as long as sources of -SH groups remain in the reduced state or are rehydrogenated as rapidly as they are dehydrogenated, there exists in the aerobic cell a reservoir of hydrogen available to rehydrogenate diketones to dienols. However, once all the available -SH groups have been converted to S-S, dienols are no longer protected from irreversible dehydrogenation, but they tend to polymerize to more and more irreversibly oxidized quinoids that may tend to catalyze further dehydrogenations.
Such a picture of "decompensated aerobic respiration", which, according to Young and Zelle (1946), is brought about by the shift of glutathione, or other sources of sulfhydryl groups, from the reduced to the oxidized condition, was revealed experimentally by Dufrency and Pratt (1947a) with evidence obtained from three different, but convergent lines of attack. This evidence was based on the use of techniques for the detection of reduced -SH or dienol groups and for the estimation of dehydrogenase and indophenoloxidase activity. These experimenters found that reagents stated to be specific for -SH groups in living cells and tissues failed to reveal such groups in the zones of inhibition, but demonstrated their presence outside the zones. This indicated a site of intense reducing activity where proteinases may operate in the building up of nucleoprotein complexes or in the denaturation of such complexes. This region of enhanced growth of bacteria was also the location of strong positive reactions for phenolic compounds and dehydrogenase activity as compared to the relative scarcity of di- and polyphenols, and the increased phenoloxidase activity in the inhibition zones. Furthermore, the fact that sharp boundaries could be demonstrated between areas of inhibition and non-inhibition suggested to these men that a threshold effect, involving sulfhydryl and dienol groups, was in operation in these regions of the assay plates.
In order to determine the distribution of aldehydes on different parts of penicillin assay plates seeded with \emph{S. aureus} or \emph{B. subtilis}, Dufrenoy and Pratt (1947a and 1948b) used Schiff's reagent and a solution of azo-benzene-phenyl-hydrazine-sulfonic acid in H$_2$SO$_4$. The results indicated that the aldehyde groups were present in the region of enhanced growth, but absent in the inhibition zone. Thus, Dufrenoy and Pratt (1948b) interpreted the over-all effect that was observed as a result of exposure of certain microorganisms to bacteriostatic concentrations of penicillin in terms of a shift of sulfhydryl (-SH) to disulfide (S-S), of aldehyde CHO to carboxylic COOH or ketonic CO, or of enolic COH to ketonic CO.

Henry and Stacey (1943) and Henry et al. (1945) reported that gram-positive organisms differ from gram-negative ones in that, on autolysis as pH 8 and 37°C, the former release some gram-positive nucleoprotein, made up of nucleic acids such as the magnesium salt of ribonucleic acid, and of a basic protein which, in its native state, involves -SH groups.

In this light, it is interesting to note that Frieden and Frazier (1947) found that a reduction in the concentration of Mg$^{++}$ considerably reduced the sensitivity of \emph{S. aureus} to penicillin. Thus, they postulated that the absence of sufficient magnesium affects either the deposition of nucleoprotein or the metabolic system involved in its
decomposition.

Dufreney and Pratt (1948a) used Pappenheim's mixture of methyl green and pyronine in order to distinguish between ribonucleic and desoxyribonucleic acid on penicillin agar plates seeded with S. aureus, B. subtilis, or P. vulgaris. The results revealed a preferential red staining of ribonucleic acid derivatives by pyronine, and blue-green staining of desoxyribonucleic acid derivatives by methyl green. The inhibition zones, therefore, were seen to be areas in which the organisms had been stripped of their basophilic (ribonucleic acid) constituents, while each zone was surrounded by a ring in which the organisms exhibited marked basophilia. This also showed that the pattern found on penicillin assay plates was due, not only to oxidation-reduction, but to the phenomenon of differential adsorption. Furthermore, the use of the redox indicator, triphenyl-tetrazolium chloride, revealed very low, if any, dehydrogenase activity within the zones of inhibition, but an intense reducing activity in the region of enhanced growth which corresponded with the basophilic areas revealed by Pappenheim's stain. Since the results were the same when ribonuclease was substituted for penicillin, these investigators concluded that penicillin, like ribonuclease, acts by stripping away or digesting the ribonucleic acid compounds, thus leaving behind the desoxyribo nucleic residues which stain with methyl green.
Massart et al. (1947a) found that penicillin acted by inhibiting the activity of ribonuclease, the substrate being the ribonucleoproteins of yeast cells. By means of trypan-flavin staining, followed by colorimetric readings, these men determined the amount of ribonuclease which resisted the attack of ribonuclease. For three of such experiments, the results are summarized in Table 4. From these results, it appears that concentrations of $1 \times 10^{-3}$ and $1 \times 10^{-4}$ of penicillin produced a strong inhibition of ribonuclease activity, whereas concentrations of $1 \times 10^{-5}$ were only slightly inhibitory.

Further work by Pandalai and George (1947) has revealed that nucleic acid appears to be of paramount importance for the growth and multiplication of organisms, such as S. aureus, Streptococcus viridans, and B. subtilis. Thus, they postulated that penicillin, in rendering these bacteria non-viable, probably acts by forming inactive complexes with nucleic acids, thus preventing the utilization of the energy material essential to the nutrition of the organisms.

Krampitz and Werkman (1947) studied the effect of penicillin on the nucleic acid metabolism of a mass inoculum of S. aureus. In the absence of penicillin, the endogenous oxygen uptake was maintained at a constant low level for five hours when rapid acceleration occurred. When sodium ribonucleate was used as the substrate, penicillin completely
TABLE 4

PENICILLIN AND RIBONUCLEASE

<table>
<thead>
<tr>
<th>Colorimetric reading (amount of RNA* having resisted the attack of ribonuclease)</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>30 min.</td>
</tr>
<tr>
<td>1. For control, without ribonuclease and penicillin</td>
<td>0.55</td>
</tr>
<tr>
<td>2. For control, with ribonuclease but without penicillin</td>
<td>0.20</td>
</tr>
<tr>
<td>3. Ribonuclease with penicillin $1 \times 10^{-3}$</td>
<td>0.35</td>
</tr>
<tr>
<td>4. Ribonuclease with penicillin $1 \times 10^{-4}$</td>
<td>0.30</td>
</tr>
<tr>
<td>5. Ribonuclease with penicillin $1 \times 10^{-5}$</td>
<td>0.25</td>
</tr>
<tr>
<td>6. Ribonuclease with penicillin $1 \times 10^{-6}$</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*RNA = ribonucleic acid
inhibited metabolic activity. The similarity between the curves representing sodium ribonucleinate and the endogenous activity suggested that the substrate oxidized in the cells was ribonucleic acid.

In order to elucidate the nature of this endogenous metabolism, the products were determined and found to be carbon dioxide and acetic acid. This relationship may be expressed by the following equation, which represents the oxidation of a pentose (source: ribonucleic acid) with a respiratory quotient of 1, and the accumulation of acetic acid:

$$C_5H_{10}O_5 + 3O_2 \rightarrow CH_3COOH + 3CO_2 + 3H_2O$$

As shown in Table 5, the ratio of oxygen uptake: carbon dioxide liberated: acetic acid produced was 3:3:1 during the endogenous metabolism. Since this same ratio existed when sodium ribonucleate was used as the substrate, the hypothesis that the endogenous activity is related to nucleic acid metabolism was further strengthened. In addition, since the desoxyribose type of nucleic acid did not decrease during metabolism, penicillin only acted on the ribose type.

These same investigators, to show that the effect of penicillin on the endogenous oxygen uptake was not general toxicity, but inhibition of a specific metabolic process, added glucose as a substrate to **S. aureus** after the lag period had continued for four hours. The presence or ab-
TABLE 5

COMPARISON OF OXYGEN, CARBON DIOXIDE, AND ACETIC ACID CONTENT DURING DISSIMILATION OF RIBONUCLEIC ACID

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Millimoles</th>
<th></th>
<th></th>
<th></th>
<th>O₂:CO₂:CH₃COOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O₂</td>
<td>CO₂</td>
<td>CH₃COOH</td>
<td>R. Q.</td>
<td></td>
</tr>
<tr>
<td>Endogenous</td>
<td>0.661</td>
<td>0.659</td>
<td>0.224</td>
<td>0.99</td>
<td>3:3:1</td>
</tr>
<tr>
<td>Sodium ribonucleate</td>
<td>1.520</td>
<td>1.531</td>
<td>0.521</td>
<td>1.01</td>
<td>3:3:1</td>
</tr>
</tbody>
</table>
sence of penicillin had no influence on the organism's activity, since, in both cases, there was an immediate accelerated oxygen uptake. However, without the addition of glucose, the acceleration of oxygen uptake occurred in a typical manner in the absence of penicillin and showed the usual inhibition in its presence. Thus, they concluded that the effect of penicillin was specific on some metabolic process of nucleic acid metabolism. Furthermore, it is possible that a specific -SH protein is being inhibited in the chain of nucleic acid metabolism. Thus, in some as yet undetermined manner, penicillin interferes with the dissimilation of ribonucleic acid and consequently with its assimilation during growth.

Dufrenoy and Pratt (1948b), in an effort to determine the enzymatic system involved in the dissimilation of ribonucleates, turned their attention to the detection of alkaline phosphates and phosphates on standard assay plates seeded with S. aureus. Upon flooding the plates with a saturated solution of triphenolphthalein phosphate (TPP), incubating them for one hour at 36°C, and then alkalizing with 1% NaOH solution (molybdate test), the zones of inhibition appeared clear on a red background. The red color developed more rapidly and was more intense in the ring of enhanced growth that surrounded each inhibition zone than in the general background. Thus, the region of increased growth was revealed as the site of the most intense alkaline
phosphatase activity.

The classical Gomori test showed that this region was also richest in phosphorous compounds. This was demonstrated by the fact that the ring of enhanced growth became black (CoS) after treatment with a 1% aqueous solution of cobaltous nitrate (which linked the phosphate to cobalt), followed by the addition of a 1% aqueous solution of ammonium sulfide. The remainder of the plate, however, failed to react.

Further work by Pratt and Dufrenoy (1949) consisted of treatment of assay plates, first with triphenyl-tetrazolium chloride, which indicates the site of high dehydrogenase activity, and then with trypan blue, which is relatively insensitive to reduction, but specifically stains ribonucleic acid inclusions. These experiments revealed the following sequence of events as penicillin diffused onto plates seeded with S. aureus: (a) sub-bacteriostatic concentrations of penicillin induced an increase in reducing activity and in staining, presumably due to ribonucleic acid materials; (b) bacteriostatic concentrations suppressed the reducing activity (no response with TPT), but permitted the cells to retain maximal ability to absorb trypan blue. This was interpreted as indicating further evidence for the inhibition by penicillin of the enzyme system (phosphatase), which is responsible for the splitting of phosphoric acid away from the ribose fraction.
Effect on Carbohydrate Metabolism

In order to determine the effect of penicillin on carbohydrate metabolism, Segalove (1947) used selected strains of staphylococci grown in glucose and lactose Amigan liquid media. Although no appreciable difference in pH took place in cultures grown in media containing glucose, with or without penicillin, the presence of the antibiotic resulted in less acid production in the lactose-containing media. These results suggested that penicillin interfered with the fermentation of lactose, and probably of other disaccharides.

Since lactose on hydrolysis yields glucose and galactose, a similar experiment was done in the presence of glucose and galactose media. The results indicated that, although the organisms grew well on both media, the cultures fermented 0.5% galactose much more slowly than 0.5% glucose. Furthermore, since penicillin was found to inhibit the fermentation of galactose, it was postulated that the presence of this antibiotic specifically interferes with galactose fermentation.

Gros and Macheboeuf (1947a) studied the action of penicillin on the carbohydrate metabolism of Clostridium sporogenes. They found that penicillin has no significant action on the glycerophosphatase activity of non-proliferating suspensions of Clostridium, and no appreciable effect on the formation of phosphoric esters in the presence
of glucose. However, penicillin did considerably inhibit the dephosphorylation of adenosine triphosphate (ATP) by these organisms. However, the same authors (1947b) pointed out that there is a difference between the action of an antibiotic on a microbe and on the muscles of mammals, since they found that, in the latter case, penicillin did not inhibit the dephosphorylation of ATP.

Effect on Fat Metabolism

Pratt and Dufrenoy (1947a) showed experimentally by evidence from standard sixteen-hour assay plates, treated with appropriate reagents, that penicillin has an effect on the fat metabolism of S. aureus. Thus, concomitant with changes in the rate of absorption of solutes and the disorganization of cellular nucleoproteins, lipids are displaced from cells undergoing lysis in the inhibition zones as a result of exposure to bacteriostatic concentrations of penicillin, and are hydrolyzed into fatty acids.

A convergent line of evidence that fatty acids are liberated at the site of bacteriolysis was furnished by flooding sixteen-hour assay plates with a saturated aqueous solution of copper sulfate and further incubating them for six hours at 37°C. A thick, opaque layer of bluish copper salts of fatty acids developed entirely over the areas of inhibition, whereas the areas of uninhibited growth did not appreciably react with the reagent.

Further support for the conclusion, drawn from the
results obtained with Nile blue and copper sulfate, was provided by experiments with FD and C yellow no. 3, a fat-soluble dye which stains neutral fat a bright yellow and imparts a deep orange color to fatty acids. Plates flooded with a saturated solution of this dye in methylal developed a bright orange color where lysis occurred, surrounded by a deep orange ring delineating the ring of enhanced growth.

Effect on Other Metabolic Activities

Pratt and Dufrenoy (1948a) have found that, under the bacteriostatic influence of penicillin, cells of S. aureus lose the ability, possessed by normal cells, to absorb silver nitrate. Thus, areas of the plate, in which the cells have lost this ability, appear clear following treatment with a solution of AgNO₃, exposure to light, and subsequent treatment with an appropriate developer. On the other hand, areas of the plate containing cells that do not absorb silver appear black. The other extremity of the inhibition zone on such plates corresponds to the site where penicillin, in its outward diffusion from the cylinder, has reached the critical concentration that causes maximal bacteriostatic effect with the shortest time of exposure. This region is that of least density of silver, and appears as a clear ring on these assay plates. Immediately outside this ring, the concentration of penicillin attained is such that it enhances metabolism and growth; in this region of increased growth the deposition of silver is greatest. Im-
mediately inside the clear periphery of the inhibition zone is a region in which, despite the fact that there is a higher concentration of penicillin, deposition of silver occurs. This indicates a less rapid impairment of cellular activity than occurs in areas where cells were exposed to lower concentrations for a shorter period of time. Thus, these men concluded that the test organisms that give the greatest response, as evidenced by physical development, are those that have been exposed for the shortest time to a minimal effective concentration, thus inducing irreversible injury to the cells.

Ungar and Muggleton (1946) observed that the addition of sub-bacteriostatic amounts of penicillin to growing cultures of Mycobacterium tuberculosis increased their rate and extent of growth. In order to show that this effect was due to penicillin and not to impurities, two samples of sodium penicillin were used: a stock penicillin having a potency of 590 μ./mg. and pure penicillin having a potency of 1650 μ./mg. These samples were added to a synthetic medium, inoculated with M. tuberculosis, and incubated at 37°C. The results, as shown in Table 6, indicate the weight and macroscopic growth of the cultures under the influence of penicillin. Since the same increase of growth was obtained with the pure and impure penicillin, these authors concluded that the increased growth was due to the effect of penicillin.
TABLE 6

WEIGHT AND MACROSCOPIC GROWTH OF CULTURES OF MYCOBACTERIUM tuberculosis UNDER THE INFLUENCE OF PENICILLIN

<table>
<thead>
<tr>
<th>Group</th>
<th>Penicillin added</th>
<th>Culture No.</th>
<th>Mean Index</th>
<th>Weight of Growth (gm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nil</td>
<td>2.0 1.0 1.5 1.0 2.0</td>
<td>1.5</td>
<td>2.3</td>
</tr>
<tr>
<td>2</td>
<td>590 µ./mg. 5.0 u./ml.</td>
<td>3.0 3.0 5.0 5.0 4.0</td>
<td>4.0</td>
<td>6.6</td>
</tr>
<tr>
<td>3</td>
<td>1.0 u./ml.</td>
<td>5.0 5.0 5.0 3.0 5.0</td>
<td>4.6</td>
<td>10.1</td>
</tr>
<tr>
<td>4</td>
<td>1650 µ./mg. 5.0 u./ml.</td>
<td>4.0 5.0 5.0 5.0 5.0</td>
<td>4.8</td>
<td>9.9</td>
</tr>
<tr>
<td>5</td>
<td>1.0 u./ml.</td>
<td>5.0 5.0 5.0 5.0 5.0</td>
<td>5.0</td>
<td>10.0</td>
</tr>
<tr>
<td>6</td>
<td>As 2 but inactivated</td>
<td>2.0 2.0 1.0 0.5 1.0</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>7</td>
<td>Penicillinase control</td>
<td>2.0 0.5 3.0 2.0 2.0</td>
<td>1.9</td>
<td>2.8</td>
</tr>
</tbody>
</table>
Similar work by Curran and Evans (1947) also showed that suitable, low concentrations of penicillin stimulated the growth of many spore-formers, such as Bacillus megatherium, and of certain non-sporogenic bacteria, such as S. aureus and Streptococcus agalactiae.

The experiments enumerated above account for or confirm, in part, the narrow ring of enhanced growth reported by Pratt and Dufrenoy on penicillin assay plates. These collaborators (1947b) suggested that this zone of increased growth probably represented a visible manifestation of enhanced metabolism, induced in cells of this region by subbacteriostatic concentrations of penicillin. However, it is also possible that bacteriostatic concentrations of penicillin, acting on cells in the areas of inhibition, cause the liberation of some cell components, especially the nucleoproteins, into the agar. From here these substances may diffuse to regions of the plate where they will serve as metabolites or growth factors.

Support for such an hypothesis was given by Bonêt-Maury and Pérault (1945), who, by the use of a recording photometer, observed that, when S. aureus was cultured in the presence of small amounts of penicillin in broth, two waves of growth occurred. They interpreted this biphasic action of penicillin as indicating that the most sensitive organisms, which were first affected by penicillin, released into the medium substances that promote a second wave of growth among
the more resistant cells.

Dufreney and Pratt (1948b) ascribed the ring of enhanced growth on penicillin assay plates containing phosphorus compounds as due to the high metabolic activity of the cells in this region. This, in turn, depleted the initially available supply of soluble phosphates, thereby creating a diffusion gradient so that phosphates were actually drawn into this area from other regions of the plate. In addition, under the effect of bacteriostatic concentrations of penicillin, the micro-organisms released into the medium organic phosphorylated compounds, which then diffused into the region of intense growth where they were utilized as growth factors. Thus, they concluded that penicillin, in addition to its bacteriostatic effect, exerts a secondary action. It increases the permeability of the most sensitive micro-organisms in the colony so that some of their constituents are released into the medium where they serve to enhance the metabolic activity and to stimulate the growth of more resistant cells.

Lee et al. (1945) believed that the action of penicillin was fissibactericidal, i.e. it killed bacteria only when they were growing or dividing. They reached this conclusion by arguing that micro-organisms, unless they were growing, would not have to share with penicillin the food that was commonly available. This mode of action would therefore offer a way of survival for a bacterial species, since any change in
conditions which would arrest fission of the micro-organisms would render them insusceptible to the antibacterial action of penicillin.

This hypothesis has been confirmed by various men. For example, Hobby and Dawson (1944) showed that conditions which increase the rate of growth of bacteria augment the rate at which penicillin acts, whereas factors which decrease the rate of growth also diminish the rate of penicillin activity:

(1) Conditions which enhance growth:

Hemolytic streptococci were incubated in plain broth, in broth containing varying concentrations of defibrinated rabbits' blood, and in whole defibrinated rabbits' blood. A constant amount of penicillin was added to each of these media, and the number of organisms per ml. was determined at various intervals. In the presence of defibrinated rabbits' blood there was an increase in the rate of growth of hemolytic streptococci and a corresponding increase in the rate of their destruction by penicillin. These results did not occur in plain broth.

(2) Conditions which inhibit growth:

When hemolytic streptococci were incubated in media containing a constant amount of penicillin and varying concentrations of physiological saline, there was a decrease in the rate of growth of hemolytic streptococci and a corresponding decline in the rate at which the organisms were
destroyed by penicillin. Therefore, these investigators concluded that penicillin is most effective when active multiplication takes place.

Miller and Foster (1944) lent further support to the idea that penicillin acts on bacteria only in an environment which promotes their multiplication. They found that the use of a non-nutrient medium resulted in an almost constant viable population of meningococci. However, in the presence of fresh blood serum, penicillin had a meningococcidal action.

Dunham and Rake (1945) observed that certain solutions of partially purified penicillin destroyed the reproductive capacity of pathogenic strains of Treponema pallidum without inhibiting their function of locomotion. For example, spirochetes exposed to 1,100 units of partially purified penicillin per ml. did not produce any demonstrable signs of disease in rabbits, although many of the organisms were still motile.

This work was confirmed by Fraxier and Frieden (1946), who found that, in the case of cultured strains of T. pallidum, penicillin was actively spirocheticidal in minute doses, although it did not affect the motility of the organisms. Thus, it appeared that penicillin may directly inhibit the function of multiplication of spirochetes without initially destroying their power of motion.

Chain and Duthie (1945) measured the oxygen uptake of
staphylococcal suspensions exposed to penicillin in different phases of their culture cycle. They found that, during the resting phase, even large concentrations had no effect on the rate of oxygen uptake. However, during the early lag phase and the logarithmic phase of multiplication, small concentrations of penicillin exerted a strong inhibitory effect and eventually stopped the oxygen uptake of the suspensions. Hemocytometer and viability counts which were run in parallel with the measurements of oxygen uptake also showed that penicillin had a strong bactericidal effect on staphylococci in the early lag and logarithmic phases. During the resting period, however, there was no measurable bactericidal effect. Thus, they concluded that penicillin interfered with a metabolic function involved in the early stages of bacterial development.

Hirsch and Dosdogru (1947) reported two types of action of penicillin which took place during the logarithmic phase of multiplication and which depended upon the concentration of the antibiotic that was used. Small quantities of penicillin (0.05-0.1 units per ml.), after a considerable latency, produced a "degenerative effect", i.e. a decrease in respiration which accompanied the disintegration of the cocci. Larger amounts (1-1,000 units per ml.), after a shorter latency period, brought about a cessation of respiration; this corresponded to a bacteriostatic effect,
in which autolysis of the cocci did not occur.

Parker and Marsh (1946) found that, whereas the ultimate lethal effect of penicillin on staphylococci was uniform, the time of onset of the effect was distinctly influenced by the concentration of the drug. Thus, when the concentration was high (1.0 unit/ml.), logarithmic growth was replaced at once by logarithmic death. On the other hand, when the concentration was reduced, a lag appeared, so that a full generation of cells was produced before bacterial death began. These men presumed that the lag before sterilization began was a period during which a critical concentration of penicillin was built up, or the supply of some essential metabolite was exhausted. Thus, under certain conditions, penicillin may act, not only on dividing cells, but after fission has taken place.

A further observation by these latter authors, that staphylococci exposed to penicillin did not immediately remultiply on removal of the drug, led Eagle (1949) to conclude that the bactericidal action in the infected animal is the sum of the direct effect of penicillin itself, plus the bactericidal action of the mechanisms of the host.

Todd (1945) proposed three possible explanations for the bacteriolytic action of penicillin on micro-organisms:

(1) Penicillin acts by killing the organisms, making them more vulnerable to autolysis. This idea was supported by the fact that pneumococci, C. welchii, and staphylococci,
which all produce powerful autolysins, are most easily lysed by penicillin within a short time. Furthermore, *S. viridans*, which produces only a small amount of autolysin, is much more resistant to bacteriolysis by penicillin, and hemolytic streptococci, which do not produce any demonstrable autolysin, are either highly or totally resistant to the lytic action of penicillin. This correlation between susceptibility to lysis by penicillin and productions of autolysins suggests that lysis occurs after the organisms have been killed by penicillin, and, thus rendered vulnerable to autolysin.

(2) Penicillin may render the organisms sensitive to autolysin without killing them, or may activate the autolysin so that it becomes effective against living organisms. Since *C. welchii* autolysin had no effect on the bacteriolytic rate of either *C. welchii* or certain staphylococci, and since anti-autolysin did not alter the bactericidal rate in penicillin cultures, this author concluded that autolysin only affects the rate of killing by penicillin indirectly, through alteration of the rate of multiplication.

(3) Penicillin may have a direct bacteriolytic action. The main evidence in favor of this view was the small amount of lysis of hemolytic streptococci which occurred after prolonged incubation in penicillin broth although these organisms do not produce any demonstrable autolysin. However, Knox (1945) failed to obtain lysis of these hemolytic streptococci by penicillin in either liquid or solid media.
Thus, Todd concluded that bacteriolysis, in cultures containing penicillin, is largely, if not entirely, due to the action of autolysins on dead organisms.

Potential measurements of various bacteria, such as *E. coli* and *B. subtilis*, which Dorfman (1944) had exposed to penicillin and other antibiotics, revealed a sudden increase in their $\zeta$ (electrokinetic) potential. A plausible explanation was that a chemical interaction between the antibiotic and bacterial surface had occurred. This, in turn, resulted in a dissociation of some ionogenic constituents of the cell surface and a rise in the $\zeta$ potential of the bacterial cell. This writer, therefore, attributed the action of penicillin to a change in the distribution of electrostatic charges at the cell surface.

**Influence of Culture Medium on Penicillin Activity**

Sulfhydryl and Amino-bearing Compounds

The hypothesis of Dufrenoy and Pratt (1947a) that penicillin exerts its bacteriostatic action by promoting dehydrogenation of $\text{-SH}$ groups to $\text{S-S}$ more rapidly than the organisms can restore the active sulfhydryl groups is in full agreement with the observation of Cavallito et al. (1945) that cysteine suppresses the antibacterial activity of penicillin. In their research, tests for antibacterial action and for inactivation of this activity were conducted with *S. aureus* by two methods, the Oxford cup test and
titration in broth. Both methods confirmed the theory that cysteine was an effective inhibitor of penicillin, and showed that the rate of inactivation of sulfhydryl compounds, such as cysteine, was a function of the time of the reaction and the concentration of the reactants. For example, the lower the concentration of penicillin, the greater the time and the excess of sulfhydryl reagent necessary to produce inactivation. They concluded that, in order to produce bacteriostasis, it would be necessary for the antibacterial substance to be concentrated in the vicinity of the sulfhydryl groups, so that reaction with these groups could occur. This concentration of the antibiotic could result from an adsorption of penicillin at the surface of an -SH active enzyme, where reaction could occur with the protein -SH groups or with -SH groups of simple molecules adsorbed by the protein.

Chow and McGee (1945) also found that the antibiotic activity of penicillin could be easily abolished by the presence of cysteine in a slightly alkaline medium. Within the pH range tested (5.3 to 7.6), they found that the rate of destruction increased with increasing alkalinity, so that about 0.04-0.08 mg. of cysteine were sufficient to inactivate 0.02 mg. of crystalline penicillin. Since they believed that the inactivation of penicillin was due to a chemical reaction, they tried but failed to inactivate penicillin by means of other amino acids, such as glycine, cystine,
methionine, and serine. Therefore, they concluded that the inactivation of penicillin by cysteine may involve both the sulfhydryl and amino groups of cysteine.

Since cysteine esters and acetylcysteine are not true models of structures present in proteins, Cavallito (1946a) prepared various sulfhydryl-containing peptides for comparison of their reactivity towards penicillin. The results, as shown in part in Table 7, indicate that the reaction of penicillin with enzyme -SH groups would be expected to take place readily only when the -SH group is part of a cysteine residue attached at the end of a polypeptide chain. This reaction would take place more slowly with cysteine -SH in which free basic groups were in a neighboring amino acid of the chain, and, least of all with a cysteine residue containing no neighboring basic amino group.

Leonard (1946) suggested that, until it can be proven that penicillin binds to -SH groups of reduced bacterial protein in proportion to the number of such groups in the protein, the actual point of attack of penicillin on microorganisms cannot definitely be concluded to be upon the thiols of the organism. Thus, it is possible that the reducing agent may be simply hydrogenating the antibiotic to an inactive substance in vitro rather than removing it from combination with thiols of the microorganism.

Although Cavallito (1946b) agreed with Leonard that it has not been proven that penicillin binds protein -SH groups,
<table>
<thead>
<tr>
<th>Thiol</th>
<th>Approximate time in hours required to inactivate Penicillin G</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-Cysteine</td>
<td>&lt;1</td>
</tr>
<tr>
<td>l-Cysteinyl-glycine</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Glycyl-l-cysteine</td>
<td>18</td>
</tr>
<tr>
<td>Glutathione</td>
<td>72</td>
</tr>
<tr>
<td>N-Acetyl-cysteine</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Thioglycolate</td>
<td>&gt;&gt;100</td>
</tr>
</tbody>
</table>
he disagreed with the statement that penicillin needs to bind such groups in direct proportion to the number of -SH groups in proteins. He reached this conclusion after he (1946a) showed that some types of -SH groups react only sluggishly or not at all with penicillin.

Support for the view that there may be a distinct difference between the penicillin-cysteine relationship in vivo and in vitro was suggested by the observations of Hirsh and O'Neil (1946). They found that inactivation of penicillin by cysteine occurs more readily in physiological saline solutions of penicillin than in the presence of blood. In order to prove this theory, 1 ml. of 6% cysteine hydrochloride was added to a group of four flasks. 2.5 ml. of penicillin G, containing 20 units per ml. were added to flasks 1 and 2. Flask 1 was made up to 40 ml. with tryptose phosphate broth, and flask 2 was made up to the same volume with tryptose phosphate blood broth. Fifty units of penicillin X plus 36.5 ml. of physiological saline solution were added to flask 3. Flask 4 was similarly prepared with physiological saline solution and blood. For each flask, 0.4 ml. was withdrawn immediately for control determinations of the penicillin content of the flasks, which were then incubated at 37°C for a period of two hours. The results showed that inactivation of penicillin was delayed and incomplete in the individual flasks containing blood and broth, or physiological saline solution and blood. However, in
flask 3, which contained physiological saline solution alone as a diluent, cysteine completely inhibited the action of penicillin. Thus, they concluded that the failure of cysteine to inactivate the penicillin was due to the antagonistic action of the broth and blood that was present.

Pratt and Dufrenoy (1948b) interpreted the fact that cysteine suppressed or reversed the action of penicillin as evidence for an indirect action of cysteine. They proposed that this amino acid, when present in low concentrations, exerted its effect, not primarily on the antibiotic agent, but rather on the cell of the test organism. Thus, in vivo, cysteine serves mainly as a source of \(-\text{SH}\) groups, so that the organism has available a reservoir of sulphydryl groups sufficient to fulfill its requirements, and, therefore, can tolerate concentrations of the antibiotic that would otherwise be toxic.

In conclusion, it is noteworthy to mention the concept of antibacterial action expressed graphically by Bailey and Cavallito (1948) in the following scheme:

\[
\begin{align*}
\text{PSH} & \not\rightarrow \text{An} \quad \xrightarrow{(1)} \quad \text{PSH}\cdot\text{An} \quad \xrightarrow{(2)} \quad \text{PSH} \not\rightarrow \text{An}' \\
\text{cysteine} & \quad \xrightarrow{(3)} \quad \text{cysteine}\cdot\text{An} \quad \xrightarrow{\text{$P'$SH} \not\rightarrow \text{An}'}
\end{align*}
\]

where PSH is the \(-\text{SH}\) active enzyme, An is the active antibacterial agent, PSH\cdot An is the enzyme-antibiotic reaction product, P'SH is the enzyme that has been dissociated from the antibiotic but altered by the reaction so as to be inert, and An' is the altered antibacterial agent.
1. A bactericidal system could be:
   a. one in which the reaction between enzyme and antibacterial agent is irreversible (reaction (1)), or
   b. one in which the antibiotic has altered the enzyme so that it is inert and is itself altered (reaction (3)).

2. Bacteriostasis may be explained by assuming that:
   a. reaction (1) is reversible, so that a concentration of antibiotic forcing the reaction to the right would lead to suspension of growth, or
   b. reaction (2) occurs.

3. The resumption of respiration and growth resulting from treatment of a bacteriostatic system with thiol compounds may be explained in two ways:
   a. the cysteine may remove the antibacterial agent from the equilibrium reaction (reaction (4)), or
   b. the thiol compound may displace the antibiotic fragment from the enzyme-antibiotic reaction product with the resulting formation of an inactive, altered antibiotic fragment and the native enzyme (reaction (2)).
Cobalt

Strait et al. (1948), in the course of their work, were led to test the effect of trace amounts of cobalt on penicillin activity in vitro. They found that the addition of CoCl₂·2H₂O, in concentrations of 0.1 to 10 parts per million, to test plates increased four to eight times the effectiveness of relatively dilute penicillin solutions in inhibiting organisms, such as S. aureus, E. coli, P. vulgaris and B. subtilis. This enhancing effect of cobalt was revealed by the pronounced increase in the diameter of the zone of inhibition, and by the shorter period of incubation that was required for the development of discernible areas of inhibition. These collaborators, therefore, believed that cobalt lowers the effective threshold concentration of penicillin. As an example of their work, ten units per ml. was the lowest concentration of penicillin that produced a detectable inhibition zone (12 mm. in diameter) on cobalt-free media with E. coli organisms. However, when the test agar contained cobalt chloride in a concentration of 0.1 mg. per liter, penicillin solutions (1 unit per ml.) produced inhibition zones with an average diameter of 15 mm. Furthermore, they observed that the duration of the contact of cobalt solutions with the organisms was important in regard to the enhancing effect of the cation. Thus, they postulated tentatively that the effect of cobalt was due, in part, to a kind of "sensitization" of the bacteria with the result
that a stimulation of metabolism and/or growth tended to place the organisms in a physiological condition in which they would be more readily susceptible to the action of penicillin.

Pratt et al. (1948) further demonstrated the enhancement of penicillin effectiveness in vivo by injecting, intraperitoneally, small amounts of cobalt along with crystalline sodium penicillin into adult mice infected with E. typhosa. They observed that 2,000 units of penicillin together with cobalt chloride (64 micrograms) protected animals for twenty-four hours as well as did 3,000 units without cobalt. Because of the demonstrated effect by Albert (1947) that cations have the capacity to form complexes with bacteriostatic agents, these authors ascribed the effectiveness of cobalt in lowering the threshold concentration of penicillin to the formation of complexes with -SH groups or some other essential component of an energy-providing oxidation-reduction system.

The pH of the Medium

Publications relating to the effect of pH on penicillin activity are contradictory. For example, Foster and Woodruff (1943a) found that, in the plate method of assay of penicillin, the pH of the assay medium had an unexpected effect on penicillin. Table 8 shows that the activity of penicillin on S. aureus H increased as a result of lowering of the pH. Furthermore, it may be observed that the
TABLE 8

EFFECT OF pH OF THE TEST MEDIUM ON THE INHIBITION OF STAPHYLOCOCCUS aureus BY THE PLATE METHOD OF ASSAY

<table>
<thead>
<tr>
<th>Penicillin (µg./ml. agar)</th>
<th>pH 5.5</th>
<th>pH 6.0</th>
<th>pH 6.5</th>
<th>pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>ƒ</td>
<td>ƒ</td>
<td>ƒ</td>
<td>ƒ</td>
</tr>
<tr>
<td>0.08</td>
<td>-</td>
<td>ƒ</td>
<td>ƒ</td>
<td>ƒ</td>
</tr>
<tr>
<td>0.12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.16</td>
<td>-</td>
<td>-</td>
<td>ƒ</td>
<td>ƒ</td>
</tr>
<tr>
<td>0.20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.24</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ƒ</td>
</tr>
</tbody>
</table>

ƒ = growth
- = inhibition
sensitivity effect of pH 5.5 was three times greater at pH 5.5 than that obtained at 7.0. This same result was noted with other sensitive bacteria including *Streptococcus hemolyticus*, *Micrococcus lysodeikticus*, *Salmonella paratyphi* A, and *Staphylococcus albus*.

Contrary to results with the plate method, Foster and Wilker (1943), by turbidimetric measurements, found that the same amount of penicillin was required to bring about a 50% growth inhibition of *S. aureus* H in nutrient broth within the pH range tested (5.0 to 8.0). Thus, changes in pH may be the result, and not the cause of bactericidal action. It is possible that incidental factors, such as a prolonged lag phase in the case of pH 5.5, diffusion effects, and the amount of the inoculum could explain the pH effect obtained by the plate method.

To complicate things even more, Garrod (1945) found that the action of penicillin in broth on *S. aureus* was progressively impaired by an increase in the acidity of the medium between pH 7.0 and 5.0. However, as mentioned previously, Chow and McGee (1945) found that, within the pH range tested (5.3 to 7.6), the rate of inactivation of penicillin increased with increasing alkalinity.

A possible explanation for the effect of changes of pH on penicillin activity was presented by Woodruff and Foster (1945). Confirming their earlier work as to the role of pH on penicillin activity, they found that *Bacillus* 164 was un-
inhibited in growth at pH 6.0 or above by several hundred units of penicillin because of the formation, under these conditions, of a penicillin-destroying enzyme, penicillinase. However, when this organism was grown at pH 5.5, where it was unable to produce penicillinase, 10 units of penicillin per ml. completely suppressed its growth. On the other hand, Morgan and Campbell (1947) found that adjustment of the pH to 5.4 gave maximal yields of penicillinase from B. cereus, whereas pH values much lower than 5.0 resulted in a loss of the enzyme with a corresponding loss of penicillin activity.

Abraham and Duthie (1946), by the use of tube dilution tests, reported that penicillin in minimal concentrations became more active as the pH of the medium fell. In fact, penicillin was twice as active at pH 6.50 as at pH 7.57. Therefore, the pH was a critical factor in determining the fate of bacterial cells, especially when the antibiotic was at the borderline of its bactericidal concentration. These investigators postulated that the effect of changes in pH on the activity of penicillin was due to a competition between the acid anions of the antibiotic and the hydroxyl ions of the culture medium.

According to Brodersen (1947), the inactivation of penicillin G in an acid medium depends upon the taking up of a hydrogen ion by the ring nitrogen atom. As a result, depending on the hydrogen ion concentration, either a positive or an ampho-ion is formed. This, in turn, is sub-
sequently transformed into an inactive substance. The entire reaction may be represented as follows:

\[
\begin{align*}
\text{(penicillin G)} & \quad \text{positive ion)} \\
HPn & \leftrightarrow HPnH^+ & \rightarrow P' \\
\downarrow & \quad \downarrow \\
Pn^- & \leftrightarrow Pn^-H^+ & \rightarrow P'
\end{align*}
\]

where P' is an inactive decomposition product which may possibly, through successive reactions, be transformed into other inactive substances.

**Origin of Bacterial Resistance to Penicillin**

There are two theories which attempt to explain the origin of bacterial resistance to penicillin: (1) metabolic adaptation theory; and (2) mutation (genetic) theory.

**Metabolic Adaptation Theory**

Among the advocates of this theory is Hinshelwood (1946), who maintains that the metabolism of the bacteria is so altered as a result of exposure to the drug, that the organism can grow in a normally inhibitory concentration of the antibiotic. Thus, resistance is an acquired characteristic, which develops through the interaction between bacteria and penicillin when the two are in contact with each other.

1. **Enzyme changes (penicillinase)**
   
   a. **Proponents**

Abraham and Chain (1940) were the first investigators to
explain the observation of Fleming (1929) that *E. coli* developed resistance to penicillin. They found in an extract of this organism a substance which destroyed the growth-inhibiting property of penicillin. The conclusion that the active substance was an enzyme was drawn from the fact that it was destroyed by heating at 90°C for five minutes and by incubation with papain activated with KCl at pH 6. Furthermore, the enzyme, which was called penicillinase, was very active at pH 6 and 9, and was presumably intracellular, since no penicillin inactivator was present in culture filtrates. These men, when they found a number of other bacteria containing an enzyme which acted on penicillin, concluded that this substance may have a function in bacterial metabolism.

Harper (1943) found that penicillin-sensitive bacteria did not grow on routine bacteriological media containing inhibitory concentrations of the drug. This inhibition was overcome, however, by the addition of a preparation of penicillinase to the media. Stable preparations of the substance, made by acetone-drying of twenty-four hour plate cultures of paracolon bacilli, showed extracellular activity, since twenty-four hour culture filtrates were found to be active. Although this investigation suggested that an enzyme was concerned, this author regarded the designation of the active agent in the paracolon cultures by the term "penicillinase" as tentative only.

Kirby (1944a), in studying this phenomenon, described
the extraction of a highly potent penicillin inactivator from seven penicillin-resistant (coagulase positive) strains of S. aureus, although no such inhibitor was present in extracts of seven penicillin-sensitive strains of the same organism. This inactivator was both extracellular and intracellular, since actively growing cultures of the resistant strains caused complete destruction of penicillin in the culture fluid, although the Seitz filtrate of the fluid contained no penicillin inhibitor.

Duthie (1944) and Ungar (1944) described a penicillinase preparation which was produced from the culture medium of a strain of B. subtilis. The latter investigator found that the free formation of penicillinase in the metabolic fluid was very marked in penicillin-resistant strains of this organism. Furthermore, the amount of the active substance was related to the strength of the surface pellicle on the culture. He also investigated different conditions of growth on penicillinase production. For example, 1% glucose in papain digest broth decreased penicillinase production as did 5-10% human serum in the medium.

Woodruff and Foster (1945) reported that the ability to destroy penicillin was a property widespread, not only among bacteria, but among yeasts, fungi, and actinomycetes. For example, two yeasts, Mycoderma valida and Debaryomyces guilliermondii were fairly effective in destroying penicillin, inactivating 68 and 81%, respectively. The fungus
Papulaspora sp. was found to destroy 800 units of penicillin within two hours. In addition, Streptomyces lavendulae and Streptomyces antibioticus produced 100% inactivation of penicillin.

Benedict et al. (1945) tested species of Serratia, Escherichia, Aerobacter, Pseudomonas, Vibrio, Flavobacterium, Proteus, and Bacillus for their ability to produce a penicillin-destroying substance. A few strains were able to initiate growth in the presence of 200 units of crude penicillin per ml. These authors isolated a chance contaminant in two penicillin-production flasks which contained an exocellular penicillinase, so potent that one part in 5000 could destroy 50-60 units of penicillin per ml. Morphological and cultural studies made on the contaminants that were isolated from the flasks showed them to be closely related to or identical with B. dereum.

Gots (1945) showed a marked correlation between resistance of an organism to penicillin and its ability to produce penicillinase. In order to show this relationship, two methods were employed: (a) the agar plate method, and (b) a broth culture method. The first method depended upon the ability of the enzyme to diffuse from actively growing organisms onto a penicillin agar medium which had been previously seeded with a very sensitive strain of S. aureus. In this way, resistant hemolytic staphylococci were streaked on the surface of the plate so that the enzyme that was
elaborated during growth could diffuse into the medium and inactivate the regional penicillin. The sensitive indicator organism grew out as a satellitic zone of colonies around the line of streak. Thus, by measuring the width of this satellitic zone and by using a series of plates containing varying concentrations of penicillin, a fairly accurate quantitative estimation of the amount of penicillinase that was produced was possible.

The second method of penicillinase detection depended upon the ability of the penicillin-destroying enzyme to diffuse into a fluid medium. Thus, supernatants obtained by centrifuging twenty-four hour broth cultures of resistant staphylococci were added in equal amounts to a penicillin dilution. The mixture was incubated for two hours at 37°C, and residual penicillin was determined by the agar cup method.

The results with the broth culture supernatants were in agreement with the plate method, i.e. the resistant strains produced a penicillin inactivator, whereas the sensitive strains failed to produce penicillinase. These men believe that Kirby's (1944) failure to demonstrate the inactivator in Seitz filtrates was due to a loss of the enzyme's activity by adsorption as a result of the filtration.

Foster (1945) found that penicillinase, prepared from B. subtilis, inactivated penicillin in the presence of bicarbonate with the concomitant evolution of CO₂. The
presumption was that a new acidic group was being liberated by the action of the enzyme on penicillin. In order to determine the nature of the acid group, Benedict et al. (1945) treated samples of sodium penicillin G with penicillinase, and carried out electrometric titrations on the inactivated solutions. Interpretation of the titration curves indicated that the pH of the acid group formed during the enzyme reaction was 4.70, and that of the acid group originally present was 2.16. In addition, to demonstrate that the acidity of the reaction mixture was not due to the enzyme preparation itself, a blank determination was made on a solution containing only water and the enzyme preparation. Titration of this solution required only a negligible quantity of alkali.

Housewright and Henry (1947a) produced penicillinase from a strain of B. cereus, incubated at 37°C in one of two media: a corn steep liquor medium, or a semisynthetic medium containing Casamino acids. In both media, the pH was maintained between 6.5 and 8.5 by the addition of 1.0 gm. of CaCO₃ per flask. Considerable variability was observed in the production of penicillinase as a result of using varying amounts of commercial sodium at different time intervals. In the corn steep medium, maximum yields of penicillinase were obtained when 200 units of penicillin per ml. of medium were added initially and again after twenty-four hours' incubation; in this case the yield reached its peak at about
ninety-six hours. In the Casamino acid medium, similar results were obtained except that the yield did not reach its peak until about 120 hours.

Henry and Housewright (1947), using the principle that the manometric assay method for penicillin depends on the formation of a carboxyl group from the ring carbonyl group of penicillin which is adjacent to the ring nitrogen, added penicillinase assaying 1:1024 to concentrations of certain purines and pyrimidines which have configurations similar to that of penicillin. Since there was no significant change in the gas phase upon the addition of the penicillinase for a period of one hour, it was concluded that this enzyme was rather specific for penicillin, and had no activity on its degradation products. Furthermore, with regard to the chemical nature of penicillinase, these authors showed that it is a protein or has a protein component essential for activity when they found that pepsin and papain, activated by cysteine individually at pH 5.0, inactivated penicillinase from 50-95%. In addition, the failure of 0.01 M potassium cyanide, $6 \times 10^{-3}$ M diethyl dithiocarbamate, and 0.15 sodium azide to inhibit the action of penicillinase indicated rather definitely that it contains no iron or copper essential for its activity.

Dietz and Bondi (1948) also studied the effect of sodium azide on both penicillinase-producing staphylococci and non-penicillinase producers. They found that, when large inocula
were used, the organisms that produced the enzyme became forty-five times more susceptible to penicillin in the presence of 0.02% sodium azide. On the other hand, the non-penicillinase producers, if affected at all, became, at most, four times as susceptible as the controls. Thus, they concluded that sodium azide has an inhibitory effect on penicillinase over and above that which was exerted against the organisms which failed to produce the enzyme.

These investigators presented two possible mechanisms for the delay of penicillin destruction by sodium azide: (a) a direct interference with penicillin activity, or (b) suppression of production of penicillinase. In order to determine which mechanism was in operation, sterile penicillinase was prepared from staphylococci by acetone-ether treatment, and stored in sterile tubes. 0.5 ml. of this material was added to flasks of 1% tryptone broth containing 0.75 units of penicillin per ml. and varying concentrations of sodium azide. Since the rate of destruction of penicillin was the same in flasks containing sodium azide as in the control flasks, these men inferred that the potentiating action of sodium azide is due to a suppression of the production of penicillinase.

Bondi and Dietz (1948) studied the susceptibility of both gram-positive and gram-negative penicillinase-producing organisms to penicillin. Although small inocula of the gram-positive strains were quite susceptible to penicillin,
large ones were less sensitive. This was due to the fact that in the latter case a more rapid multiplication of the cells occurred which, in turn, resulted in greater production of the enzyme and a subsequent rapid destruction of penicillin. In addition, they found that the degree of resistance of a gram-positive organism to penicillin depended, not only upon its ability to produce large amounts of penicillinase, but upon its ability to produce this enzyme rapidly.

As evidence, assays were made for residual penicillin at various intervals, shortly after a series of tubes containing twofold concentrations of penicillin had been inoculated with *Staphylococcus* 161, *B. megatherium*, and *B. cereus*. The results of the assays from the tubes containing 5.0 units of penicillin per ml. one, two, and four hours after inoculation are shown in Table 9. It is evident that there is a correlation between resistance of gram-positive organisms to penicillin and the rapidity or speed of antibiotic destruction. *B. megatherium*, the most susceptible of the organisms that were studied, destroyed relatively small amounts of penicillin during the short intervals tested, whereas *B. cereus*, resistant to penicillin, rapidly destroyed the 5.0 units present.

These men, in studying gram-negative bacilli, found that the inoculum size did not influence their susceptibility to penicillin, since small inocula as well as large ones were
## TABLE 9

**THE RELATIONSHIP OF PENICILLIN-SUSCEPTIBILITY TO SPEED OF PRODUCTION OF PENICILLINASE**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Inoculum 0.1 ml.</th>
<th>Inhibiting conc. units/ml.</th>
<th>Penicillin-5.0 units/ml. broth Units/ml. left</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Undiluted</td>
<td></td>
<td>1 hr.</td>
</tr>
<tr>
<td>S. 161</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Undiluted</td>
<td>&gt;10.0</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>5.0</td>
<td>4.9</td>
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<tr>
<td></td>
<td>10^{-4}</td>
<td>.4</td>
<td>5.2</td>
</tr>
<tr>
<td>B. megatherium</td>
<td>Undiluted</td>
<td>1.28</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>.02</td>
<td>5.0</td>
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<tr>
<td></td>
<td>10^{-4}</td>
<td>&lt;.02</td>
<td>5.3</td>
</tr>
<tr>
<td>B. cereus</td>
<td>Undiluted</td>
<td>&gt;10.0</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>&gt;10.0</td>
<td>4.80</td>
</tr>
<tr>
<td></td>
<td>10^{-4}</td>
<td>5.0</td>
<td>5.30</td>
</tr>
</tbody>
</table>
not susceptible to this antibiotic. Therefore, they deduced that, although penicillinase production contributes to the resistance of these organisms, some other basic factor common to most gram-negative organisms is primarily responsible for their resistance.

Spink and Ferris (1945) also believed that the fundamental question was not whether a given strain produces penicillinase, but how potent and how quickly is the enzyme produced by a strain in relation to the concentration of penicillin and the time necessary for the antibiotic to destroy the cells. The significance of these two factors, i.e. potency of penicillinase utilized in a given observation, and period of time in which the enzyme is in contact with penicillin, was illustrated by the following experiments, using penicillin-resistant staphylococci:

1. Preparations of strain Bernando II in concentrations of 1 mg. per ml. and 0.1 mg. per ml. were mixed with 100 units of penicillin/ml. in physiological saline solution and permitted to stand at room temperature for five hours. At the end of this period, the penicillinase was removed by filtration and the filtrate tested for penicillin activity. There was only a slight diminution in the potency of penicillin which had been in contact with 0.1 mg. of dried cells per ml. On the other hand, there was almost complete destruction of activity with the mixture containing 1 mg.
per ml. This showed that the degree of resistance is related to the potency of penicillinase produced by a given strain.

2. To show the effect of time on penicillinase activity, 0.2 mg. of cells/ml., extracted from strain Rosen, was mixed with penicillin and tested for antibiotic activity at the end of one and three hours. Although there was no loss of penicillin activity in one hour, its action was considerably reduced in three hours.

It is interesting to mention the work of Housewright and Henry (1947b), who studied the effect of antipenicillinase, an antibody produced in rabbits, on resistant organisms which produce penicillinase. Since it has been shown by Kirby (1944a) that penicillinase may be produced intracellularly and, in certain instances, extracellularly, the question arose as to whether the antipenicillinase would be effective in both cases. Table 10 shows that the sensitivity to penicillin was increased by the antipenicillinase only in those organisms which produced extracellular penicillinase. These men postulated that the failure of antipenicillinase to increase the sensitivity of those organisms, in which only intracellular penicillinase was found, was due to the inability of the large molecules of antipenicillinase to pass through the cell membrane. In those cases where antipenicillinase was effective, these authors theorized that the combination of antigen (penicillinase) and antibody (anti-
TABLE 10
THE EFFECT OF ANTIPENICILLINASE ON THE SENSITIVITY OF BACTERIA TO PENICILLIN

<table>
<thead>
<tr>
<th>Organisms tested</th>
<th>Minimal inhibiting concentration of penicillin in µ/ml. in the presence of Normal rabbit serum 1:14</th>
<th>Immune rabbit serum 1:14</th>
<th>Extracellular penicillinase</th>
<th>Intracellular penicillinase</th>
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<tr>
<td>Penicillin resistant</td>
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<td></td>
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<tr>
<td>S. aureus, Long</td>
<td>30</td>
<td>15</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>E. typhosa</td>
<td>50</td>
<td>50</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td>25</td>
<td>25</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>E. cereus, B-569</td>
<td>100</td>
<td>50</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Penicillin susceptible</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus, Long</td>
<td>0.15</td>
<td>0.15</td>
<td>-</td>
<td>**</td>
</tr>
<tr>
<td>S. aureus, 612</td>
<td>0.08</td>
<td>0.08</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td>0.50</td>
<td>0.50</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>B. anthracis, 99</td>
<td>0.04</td>
<td>0.04</td>
<td>-</td>
<td>?</td>
</tr>
</tbody>
</table>

* Reported by Bondi and Dietz (1944)  ** Reported by Spink and Ferris (1945)
penicillinase) prevented destruction of penicillin.

Duthie (1947) observed that the formation of penicillinase by organisms of the mesentericus-subtilis group was adaptive, and depended on the presence of active substrate (penicillin), added continuously to the culture in order to produce optimal yields of the enzyme. However, the addition of penicillin to resting cells, subsequently shaken, did not result in a great increase in the yield of penicillinase. Therefore, they concluded that there is no permanent adaptation transmissible to the daughter cells.

(b) Adversaries

Too many inconsistencies are found in the literature to permit the acceptance of enzyme (penicillinase) formation as the sole factor in explaining the origin of bacterial resistance to penicillin. For example, Abraham and Chain (1940) found that M. lysodeikticus, an organism sensitive to the action of penicillin, produced penicillinase. Therefore, they postulated that the presence or absence of the enzyme, penicillinase, was not the only mechanism determining bacterial resistance or sensitivity to penicillin.

This same conclusion was reached by Bondi and Deitz (1944), who observed that certain penicillinase-negative organisms, such as S. aureus, E. typhosa, and Brucella melinensis, were not susceptible to penicillin.

Furthermore, Spink and Ferris (1945) found that, whereas penicillinase was present in four strains of staphylococci
that were made resistant to penicillin in vivo, this enzyme was not found in four other strains, made resistant in vitro. In addition, the in vivo resistance was a more permanent characteristic of staphylococci than that acquired in vitro. Thus, they deduced that the production of penicillinase was not the only reason for the development of resistance to penicillin.

Abraham et al. (1941) also showed that the adaptation of the staphylococcus to penicillin did not necessarily depend on the production of a penicillin-destroying enzyme. This was proven by growing an adapted strain of S. aureus on nutrient agar in two batches of twenty-rolled Winchester bottles. This was done in order to obtain a sufficient amount of bacterial bodies to be used in the crushing mill of Booth and Green (1938). After the growth was ground in the crushing mill for three hours, the extract was centrifuged for twenty-minutes. One ml. of this substance was then incubated with one mg. of penicillin for five hours. Since there was no sign of a loss of penicillin activity after this period, these men concluded that the resistance of staphylococci to penicillin is not due to the appearance of penicillin-destroying substances.

Treffers (1946), in studying the potentiation of penicillin by various agents, found that sodium azide, iodoacetic acid, gentian violet, and many other compounds were able to enhance the action of penicillin without involving the in-
hibition of penicillinase as an essential step. He drew this conclusion as a result of the following experimental results:

(a) low concentrations of iodoacetic acid were not able to bring about a reduction in the activity of penicillinase in experiments done with cell-free filtrates of *E. coli*, or with soluble penicillinase from a gram-positive spore-former;

(b) iodoacetic acid and other agents, however, were still capable of exerting their potentiating effect on penicillin even with *Shigella ambigua*, an organism which gives no indication of penicillinase production. Thus, they proposed that the presence or absence of penicillinase is not necessarily indicative of the origin or source of bacterial resistance.

Frieden et al. (1947) also determined that the resistance of four penicillin-fast strains of *S. aureus* was not due to the production of penicillinase. Using a method essentially similar to that of Gots (1945), these experimenters uniformly inoculated a penicillin-sensitive organism on agar plates, containing concentrations of penicillin just sufficient to inhibit the growth of the organism. Then, the bacterium to be tested for penicillinase was streaked or spotted upon the plate. After incubation, production of penicillinase was indicated by satellite growth of the sensitive form around colonies of the resistant organism. The use of penicillin concentrations ranging from 0.02-0.10 units per ml. showed
no evidence for penicillinase production by any of the four organisms that were tested.

(2) Other metabolic changes.

Abraham et al. (1941) found that penicillin-resistant staphylococci, obtained after serial passage on penicillin-containing media, showed a reduction of growth velocity in nutrient broth and agar, and a delay in hemolysis on blood-agar plates. In addition, the adapted strain showed a considerable lag in acidifying lactose and mannitol, as well as a depression in its ability to coagulate milk.

Klimek et al. (1948), in studying the increase in resistance of *S. aureus* to penicillin, observed a gradual decrease in all the fermentative properties characteristic of the parent strain. In addition, pigmentation was first reduced and then was completely lost, hemolysis was retarded, and the coagulase reaction was negative in the single test that was conducted. In contrast to the situation with streptomycin, a complete morphological and tinctorial change was observed: the culture became highly pleomorphic, showing elements varying from cocci to coccibacilli and occasional diphtheroid forms.

Bellamy and Klimek (1948a) developed a penicillin-resistant variant of *S. aureus* 209 P, which grew readily in the presence of 4 mg. of penicillin per ml. Aerobically, this resistant strain grew about one-half as fast, and reached about eight-tenths the final turbidity of the
sensitive, parent culture. However, anaerobically, there was no appreciable rate of growth of this variant. These results led these authors to propose loss of anaerobiosis as a possible cause for the slower growth of the penicillin-resistant variant. Thus, organisms that depend upon anaerobic processes for their energy supply will not develop appreciable resistance to penicillin.

These same authors (1948b) further added to the impression that penicillin-resistance involves a profound alteration of metabolism, when they observed that S. aureus lost its power to produce acid from lactose, maltose, sucrose, mannitol, and galactose. Other biochemical changes included the inability to reduce nitrates to nitrites, and to grow in 6.5% NaCl. All these characters suggested a relative loss of enzymatic abilities rather than a gain, such as is found with respect to amino acid synthesis.

Gezon (1948a, 1948b, and 1948c) studied alterations in the metabolism of Group A, B and C beta-hemolytic streptococci after they had acquired resistance to penicillin. All three groups showed changes in hemolysis from beta to alpha or gamma, when they were grown on maximal concentrations of penicillin. In addition, the group specific antigen (precipitinogen), which was demonstrable in all the controls, was present in only about one-half of the resistant strains.

Shwartzman (1944 and 1945) suggested that the refractoriness of gram-negative bacilli may result, in part,
from the interference of bacterial metabolites with the action of penicillin. In order to investigate this assumption, he studied the effect of various amino acids in synthetic media upon the inhibition of *E. coli* by different concentrations of penicillin. The results showed that dl-methionine and mixtures of dl-methionine and penicillin were toxic to *E. coli*, cultured in plain broth. However, glycine, histidine, phenylalanine, and serine produced a somewhat variable antagonistic effect upon the minimal inhibitory dose of penicillin (1 0.U. per ml.). Glutamic acid possessed a greater but also variable anti-penicillin activity. In addition, this investigator found that asparagine, an optically active natural base, showed a distinct antagonism against penicillin. For example, 2 mg. per ml. of this substance completely antagonized the effect of 1 0.U. of penicillin. Therefore, it is possible to conclude that resistance of these gram-negative bacilli was due to metabolic adaptation.

Gale (1947b) has shown that staphylococci, trained to a resistance of 2,000 Oxford units per ml. by serial sub-cultivation in increasing concentrations of penicillin, have less and less capacity to concentrate free glutamic acid. This suggests the possibility that the resistant organisms, when growing in very high concentrations of penicillin, adopt a different form of amino acid metabolism.

A still more far-reaching alteration of metabolism, which tended to link penicillin-resistance with amino acid
utilization, has been noted by Gale and Rodwell (1948 and 1949). They compared the amino acid metabolism of two strains of *S. aureus* rendered resistant to high levels of penicillin with that of penicillin-sensitive strains. The resistant organisms did not differ markedly from the parent strains in their ability to oxidize amino acids. However, they behaved like gram-negative organisms since they were able to synthesize all their amino acid requirements from ammonia and glucose in the presence of thiamine, although they had no power to assimilate free glutamic acid in their internal environment. Thus, they concluded that the more synthetically competent the organism is with regard to amino acids, the greater is its resistance to penicillin.

**Mutation (genetic) Theory**

Two of the proponents of this theory, Demerec (1945a) and Luria (1947) maintain that, within any bacterial population, individuals will be found that possess varying degrees of susceptibility to a given antibiotic. There are, however, a few organisms present that are less susceptible than the majority. In the presence of the antibiotic, the majority of the population is inhibited, and only those few resistant forms or mutants can develop. Thus, they believe that resistance is an inherited characteristic which originates through mutation, and whose origin is independent of contact with the antibiotic.
Experimental evidence by Demerec (1945a and 1945b) indicates that penicillin acts only as a selective agent which eliminates the sensitive bacteria, and, thus, allows the resistant mutants, which are always in any large population, to multiply. All experiments were done in vitro in which strains of S. aureus were first grown in broth cultures without any penicillin. These organisms were used only in the resistance tests, made by growing the bacteria on broth agar plates containing various concentrations of penicillin. This investigator found that, on low concentrations, all bacteria survived and formed colonies, but in the region of higher concentrations, survivors continued even at the highest concentration used in the experiments.

This same author considered two alternative possibilities with respect to the mechanism of this resistance: (1) resistance was induced by some interaction between penicillin and the bacteria, or (2) resistance originated by mutation with the antibiotic acting only as a selective agent which destroyed the sensitive bacteria and thus isolated the mutants.

A relatively simple method was available for distinguishing experimentally between these two possibilities. From the same broth dilution, containing about 300 bacteria per ml., 30 small test tubes were prepared each with 0.3 ml. of material, and one large tube with about 15 ml. of the material. At the same time, 20 samples of 0.3 ml. each, from
the same dilution, were plated in a medium containing 0.064 unit of penicillin per ml. in order to determine if any of the samples contained resistant bacteria. Since no resistance was observed, it was reasonable to assume that each culture was started with an inoculum consisting of susceptible bacteria only.

Cultures were then incubated at 37°C for about eighteen hours, during which time the number of bacteria increased to about $2.3 \times 10^8$ per ml. The entire contents of each of the thirty small tubes were plated in a Petri-dish with 0.064 unit of penicillin per ml. Twenty samples of 0.3 ml. each were taken from the large tube and were similarly plated in a medium containing the same concentration of penicillin.

Thus, in each of these fifty platings, about $6.6 \times 10^7$ bacteria were placed in media containing an identical concentration of penicillin. Therefore, if resistance originates through interaction between the bacteria and penicillin, then one would expect to find on each plate a similar number of resistant colonies. However, if resistance originates through mutation, then one would expect that the twenty samples, taken from the same culture, would give similar numbers of resistant colonies, while an appreciable degree of variation in the number of resistant colonies would be expected among samples from different cultures.

The results, summarized in Table 11, show that a very
**TABLE II**

NUMBER OF BACTERIA (STAPHYLOCOCCUS aureus) RESISTANT TO A CONCENTRATION OF 0.064 OXFORD UNITS OF PENICILLIN PER ML. OF AGAR MEDIUM IN SAMPLES TAKEN FROM A SERIES OF INDEPENDENT CULTURES AND SIMILAR SAMPLES TAKEN FROM A SINGLE CULTURE WHICH ASSAYED $2.3 \times 10^5$ BACTERIA PER ML.

<table>
<thead>
<tr>
<th>Culture no.</th>
<th>No. of resistant bacteria</th>
<th>Culture no.</th>
<th>No. of resistant bacteria</th>
<th>Sample no.</th>
<th>No. of resistant bacteria</th>
<th>Sample no.</th>
<th>No. of resistant bacteria</th>
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<tr>
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<td>16</td>
<td>37</td>
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</tr>
</tbody>
</table>

Average... 122.2  Average... 28.9
Variance... 46327.0  Variance... 39.8
Chi-square... 5281.0  Chi-square... 22.7
p... 0.3
slight variation exists in the number of resistant colonies among the twenty samples taken from one culture. On the other hand, the number of resistant colonies in each sample taken from the independent cultures varied greatly. This proved that resistance of *S. aureus* to certain concentrations of penicillin originated through mutation.

A very interesting feature of bacterial resistance to antibiotics is the stepwise increase in the degree of resistance that can be brought about by selection. Demerec (1945a, 1948, and 1949) showed that the building up of resistance to penicillin follows a definite pattern, and is more rapid with each selection step. For example, the first-step resistant mutants are very uniform in their degree of resistance, which was only slightly higher than that of the original strain. Additional mutations occurring in such first-step mutants allow bacteria to possess a higher (second-step) degree of resistance. In a similar manner, third-step and still higher resistant strains may be developed. In any case, the variation in the degree of resistance among mutants of the same step was slight. Thus, higher resistance may be attained only in successive steps, and no step may be skipped in the process.

On the basis of work done by Bonner (1946), who showed, by the induction of mutations with X-rays and ultraviolet radiation, that there is a genetic control of biochemical reactions in *P. notatum-chrysogenum*, Demerec (1948 and 1949)
postulated that mutations in bacteria are caused by genes. The complexity of behavior observed in the study of resistance to antibiotics suggested the idea that several genes govern the reactions that determine sensitivity or resistance. In the case of penicillin, all genes affecting resistance to penicillin have a similar potency, so that the effect of mutation is the same regardless of which of the genes happened to mutate. According to this hypothesis, there is still present in a first-step resistant strain a number of unmutated genes that affect resistance. Mutation of any of these genes produces a second-step resistant strain, which possesses a higher degree of resistance than the first-step strain. Similarly, by mutation of another gene in a second-step resistant strain, a still higher degree of resistance is attained, characteristic of the third-step resistant strain. Thus, by further repetition of the process, a very high degree of resistance may be reached.

In a follow-up of Demerec's work, Luria (1946) indicated that there are two types of penicillin-resistance in staphylococci, namely, resistance that develops as a result of mutation and which is generally permanent, and resistance that is associated with penicillinase-producing strains. However, he discounted the role of penicillinase in the mechanism of penicillin resistance by emphasizing the fact that, if a small inoculum of organisms is used in the in vitro test for sensitivity, the individual cells of resistant and penicil-
Linase-producing strains were sensitive to small concentrations of penicillin.

Miller and Bohnhoff (1945a and 1945b) found that the development of resistant gonococci and meningococci proceeded faster when transfers consisted of heavy seedings from media containing concentrations of penicillin which permitted fairly abundant growth rather than from the highest concentration which allowed only a few cells to develop. Thus, these authors (1947a) postulated that penicillin-resistance does not develop through the direct action of penicillin on the bacteria, but is the result of the selective bacteriostatic action of penicillin, which inhibits the propagation of all but the resistant variants that constantly arise by mutation.

Eriksen (1946) showed, by a modification of the agar cup method, that a penicillin-resistant staphylococcal culture consists of a mixture of rapidly growing penicillin-sensitive bacteria, represented by completely clear inhibition zones, and slowly growing resistant bacteria, which covered these inhibition zones with a luxuriant growth. Therefore, he concluded that resistance develops by a mutation in connection with the elimination of the non-resistant (sensitive) individuals.

In an effort to harmonize the metabolic adaptation and mutation theories, Segalove (1947) studied the natural and artificial resistance of staphylococcal strains to penicillin.
Thus, two naturally resistant strains (147 and 196), two artificially resistant ones (161R and 43R), and the susceptible parent cultures (161 and 43) were tested in order to determine their mechanism of resistance to penicillin.

Although penicillinase was not detected in strain no. 43R, a very high proportion of the cells were resistant to penicillin. Furthermore, the counts in plates containing penicillin were comparable to those in the control plates even when the inocula contained few bacteria, thus indicating a relative homogeneity in resistance to penicillin. Therefore, it appeared that resistance was due, in part, to selection of resistant cells by growth in penicillin-containing media.

However, the results in experiments with strains no. 161R and 147 seemed to indicate another mechanism of resistance. In strain no. 161R, an unaccountable number of colonies grew on penicillin plates inoculated with large quantities of organisms. On the other hand, the plates inoculated with the next higher ten-fold dilution of cells showed no growth. Similar tests with strain no. 147, a naturally resistant culture, gave the same type of result. The colony count seemed to indicate the production of a penicillin-inactivating substance which was present in amounts sufficient to allow growth of non-resistant cells.

The presence of penicillinase was determined by noting the growth in tubes containing sterile filtrate with penicil-
lin, and comparing them with the control tubes containing the same concentration of penicillin. Whereas growth occurred in tubes containing strains no. 161R and 147, there was no penicillinase production in strains 43R, 43, and 161.

Thus, it appears that resistance to penicillin may be due to one of two factors acting singly or in combination: (1) resistant strains may be developed through selection by using penicillin to eliminate non-resistant cells, and/or (2) resistance may be developed by means of a penicillin-destroying enzyme, penicillinase.
STREPTOMYCIN

Background

Schatz et al. (1944) noted that, before the discovery of streptothricin and streptomycin, most of the antibiotics that were known, including penicillin and other mold products, acted largely upon gram-positive bacteria. For example, penicillin was found to be highly selective in its action within the gram-negative group, affecting organisms of the genus Neisseria, but showing little activity upon E. coli and other gram-negative bacteria.

In a search for antagonistic organisms that are active against gram-negative bacteria, and from which antibiotic substances could be isolated, the actinomycetes were found to offer extensive potentialities. The organism, S. lavendulae, which produces streptothricin, was found by Waksman and Woodruff (1942) to exert a marked inhibitory effect against many of the gram-negative types of bacteria. Then, another organism, Streptomyces griseus, which produces streptomycin, was shown to have an even greater effect on various gram-negative bacteria by Schatz, Bugie, and Waksman (1944).

Structure
(Chemical Formula)

In order to understand fully the mode of action of streptomycin, it is first necessary to know its structural
formula. According to the experimental results of Brink et al. (1945), streptomycin has the general structure of an hydroxy-1-ated base (streptidine) linked glycosidically to a nitrogen-containing disaccharide-like molecule (streptobiosamine), which has a free or potential carbonyl group and a methyl-amino group:

\[
\begin{align*}
&\text{Streptidine portion} \\
&\text{(OH- base)} \\
&\text{Streptobiosamine portion} \\
&\text{(Carbohydrate-like substance)}
\end{align*}
\]

Carter et al. (1947) determined more specifically the structure of streptomycin. It is a basic glycoside, which consists of two main portions: (a) A streptidine molecule (base) which is one of the eight meso forms of 1,3-diguanido-2,4,5,6-tetrahydroxycyclohexane, and (b) a streptobiosamine which consists, in turn, of two parts: a N-methyl-l-glusosamine portion and a streptose or streptosonic lactone fragment. Streptidine is linked to the first carbon atom of streptose, but the point of attachment of streptobiosamine to streptidine is questionable.

Carter et al. (1947) maintain that this attachment is by means of the 5 carbon of streptidine, although the 4 carbon
is not entirely disregarded. Kuehle et al. (1947) suggest that the linkage is only at carbon atom 4, and illustrate the points of connection of the various fragments of the streptomycin molecule as follows:

\[
\begin{align*}
\text{Streptidine molecule} & \\
\text{Streptose or streptosonic lactone fragment} & \\
\text{N-methyl-1-glucosamine fragment} & \\
\text{Streptobiosamine molecule} & \\
\end{align*}
\]

Potential Active Groups

Three groups in the streptomycin molecule appear to be essential for streptomycin activity: (a) the guanidine groups; (b) the free amino groups; and (c) the carbonyl group.

(a) The Guanidine Groups

Carter et al. (1945) maintained that the guanidine groups were active groups of streptomycin. The presence of
this group is indicated by the fact that alkaline hydrolysis results in the formation of ammonia and the disappearance of the Sakaguchi test. In these experiments, the crude streptomycin chloride was subjected to several colorimetric tests in order to detect functional groups. Positive tests were obtained due to impurities, but the Sakaguchi test for the guanidine group was positive in all active fractions. Therefore, they concluded that the guanidine group was an active group.

Gray and Birkeland (1947), on the other hand, claimed that the streptosonic lactone fragment was the critical group of the streptomycin molecule, since they found that streptidine and N-methyl-1-glucosamine neither inhibit E. coli nor interfere with the action of streptomycin in E. coli. This refutes the idea that the guanidine groups of the streptidine fraction of streptomycin are active groups.

Cohen (1947) gave added proof that the diguanido portion of the streptomycin molecule was inactive when he found that streptidine, unlike the entire streptomycin molecule, could not react with phosphorylated nucleic acids to produce polymeric compounds.

(b) The Free Amino Groups

Fitzgerald (1948) presented evidence to show that the free amino groups of streptomycin were essential for antibiotic activity. This conclusion was based on experiments done with M. tuberculosis 607 and Myobacterium lacticola,
which were grown in the Tween medium of Dubos at 37°C. It was found that 1.0 mg. of potassium cyanate, a reverser of streptomycin activity, prevented the inhibition of enzyme formation by 0.2 mg. of streptomycin. Since the potassium cyanate alone inhibits the oxygen uptake under these conditions, it probably is not metabolized, but reacts with the streptomycin molecule, specifically with the free amino groups because of the ability of cyanate to react with amino groups. He concluded, therefore, that the free amino groups of streptomycin were necessary for antibiotic activity.

The work of Gray and Birkeland (1947) also refutes this theory since, if the streptose portion is the active part of the streptomycin molecule, then the free amino groups cannot be essential for antibiotic activity.

(c) The Carbonyl Group

According to Donovick et al. (1946), a comparison of the absorption spectra of the phenylhydrazones of streptomycin and also of the thiosemicarbazones, which are particularly useful since they can be recognized in solution by their intense absorption bands in the ultraviolet region, gives us evidence that streptomycin contains a carbonyl group (aldehyde or ketone) capable of reacting with great rapidity with such reagents as semicarbazide, hydroxylamine, and phenylhydrazine. For example, when 1.0 mole of streptomycin-hydrochloride and 1.5 moles of thiosemicarbazide are dissolved in water, and the ultraviolet spectrum of the mix-
ture is determined after fifteen hours' standing at room temperature, a band with a maximum at 270 μ is found. The position and intensity of this band show that the light-absorbing entity is a thiosemicarbazone. Furthermore, the ease with which the reaction proceeds indicates that the reactive carbonyl group is present in streptomycin as such and not in combined form.

Other experiments by this same group show that, of four compounds, semicarbazide, thiosemicarbazide, hydroxylamine hydrochloride, and hydrazine hydrate, the thiosemicarbazide is the most effective in inactivating streptomycin. In addition, they reported that, in general, carbonyl reagents played a smaller role in inhibiting bacterial growth in tryptone broth than in thioglycolate broth.

Gray and Birkeland (1947), in direct contradiction to Donovick et al. (1946), gave evidence to show that the carbonyl group is not important in the fundamental mode of action of streptomycin. They undertook this problem after they previously found that cysteine and hydroxylamine formed additional compounds on the free carbonyl group of the streptone portion of the streptomycin molecule, which led to inactivation of the fraction. In comparing these results with dihydrostreptomycin (formed by reducing the free aldehyde group of the streptose fragment of streptomycin to an alcohol) they found that the latter possesses essentially
the same activities as does streptomycin; this fact was confirmed by Fried and Wintersteiner (1947) and by Donovick and Rake (1947). However, dihydrostreptomycin, unlike streptomycin, was not inactivated by hydroxylamine or cysteine. In addition, when Gray and Birkeland (1947) found that strains of *E. coli* were equally resistant to both streptomycin and dihydrostreptomycin, they reasoned that the carbonyl group is not necessary for the action of streptomycin.

To definitely confirm the conclusion of Gray and Birkeland (1947), it is necessary to determine whether the biological activity of dihydrostreptomycin is an inherent characteristic of the compound per se or whether a conversion to streptomycin occurs before antibiotic action is possible. The evidence indicates that dihydrostreptomycin acts through a conversion to streptomycin since Schenck and Spielman (1945) by treatment with milk alkali, were able to form maltol from streptomycin, while Peck et al. (1946), Fried and Wintersteiner (1947), and Donovick and Rake (1947) failed to produce maltol from dihydrostreptomycin with this treatment. Maltol could be detected by observing its characteristic ultraviolet absorption, or, according to Boxer et al. (1947), by a phenol reagent or ferric complex.
Therefore, Gray and Birkeland are not justified in comparing the activity of these two compounds, and then arguing to the unimportance of the carbonyl group for streptomycin activity.

Effect on Morphology (Cytology) of Bacterial Cell

Effect of Metabolic Products of Actinomycetes (In General)

Borodulina (1935), in studying the antibiotic substances produced by actinomycetes, showed that the metabolic products of actinomycetes were able to deviate the normal development of Bacillus mycoides. In the first place, they delay cell division with the result that the cells of *B. mycoides* become elongated, reaching enormous dimensions and assuming most peculiar forms. In addition, spore formation is absent in cultures with a high concentration of these toxic substances, and the ammonifying capacity of *B. mycoides* is greatly reduced. However, delayed nonspore-forming variants are produced with a modified type of growth on nutrient media. Borodulina's work on the effect of a culture filtrate of Streptomyces sp. on the morphology of *B. mycoides* is summarized by Waksman (1947) in Table 12.

Effect of Streptomycin per se

Little attention has been directed towards possible alterations in bacterial morphology as a result of growth in the presence of streptomycin. Welch et al. (1946) observed definite huge and bizarre forms of *E. typhosa* in cultures
### TABLE 12

**INFLUENCE OF CULTURE FILTRATE OF STREPTOMYCIES sp. ON MORPHOLOGY OF BACILLUS mycoides**

<table>
<thead>
<tr>
<th>Days of Incubation</th>
<th>Morphology of Antagonized Bacterium</th>
<th>Macroscopic Growth in Broth</th>
<th>Spore Formation</th>
<th>Rod Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Medium plus 10% culture filtrate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Long filaments</td>
<td>x</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>4</td>
<td>Filaments have divided into elongated cells</td>
<td>x</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>17</td>
<td>Cells altered</td>
<td>x</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>45</td>
<td>Cell fragments of various shape and length</td>
<td>x</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Medium plus 5% culture filtrate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Elongated cells</td>
<td>x</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>4</td>
<td>Elongated cells</td>
<td>x</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>17</td>
<td>Greatly deformed cells</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>45</td>
<td>Greatly deformed cells</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td><strong>Control medium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Deformed cells rare</td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>/</td>
<td>-</td>
<td>/</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>45</td>
<td></td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>

* x indicates growth of *B. mycoides* in the shape of fluffy small balls inside liquid
exposed to streptomycin. However, Miller and Bohnhoff (1946) found no morphological changes in the individual cells of meningococci or gonococci grown in the presence of streptomycin.

A more detailed study by Strauss (1947) has revealed that the effect of streptomycin on the size and shape of bacteria was variable, being marked in some gram-negative bacilli, slight with gram-positive cocci, and absent with gram-positive bacilli. In the actual tests, $10^{-2}$ dilutions of overnight growths of various cocci and bacilli in broth were incubated at $37^\circ C$ in heart infusion broth in the presence of various amounts of streptomycin, which were expected to be slightly, moderately, or strongly bacteriostatic. At 0, 3, and 5 to 6 hours' incubation, the tubes were centrifuged for twenty minutes, and gram-stained smears of the bacterial sediment were prepared.

The most extreme alterations in appearance were found with the strain of *Shigella sonnei*; cells of this organism, when exposed to streptomycin, became elongated up to three to four times as long as the control cells and appeared swollen. These alterations were more pronounced with a slight bacteriostatic amount of streptomycin (1 $\gamma$ per ml.) than with a higher, stronger bacteriostatic concentration (5 $\gamma$ per ml.). Cells of the strain of *Salmonella typhimurium*, when exposed to streptomycin (3 and 6 $\gamma$ per ml.), became swollen and elongated, and assumed coccoid forms. Definite,
but lesser, degrees of alteration in cellular appearance were observed with strains of Aerobacter aurogenes and Proteus ammoniae, while the other gram-negative organisms exhibited little or no alteration in appearance. The gram-positive organisms that were studied, such as S. aureus and Micrococcus tetragenus, showed no consistent changes in shape or size as a result of incubation with streptomycin. Since Strauss observed that the mode of action of streptomycin on non-multiplying bacteria was essentially similar to its action on multiplying bacteria, he suggested that the observed morphological changes may be secondary effects resulting from disturbances of intermediary metabolism rather than primary effects on bacterial fission.

Smith and Waksman (1947) used a modification of the Ziehl-Neelsen stain in order to study the effect of streptomycin on the morphology and acid-fastness of certain tubercle bacilli. They found that the principal effects of streptomycin on the morphology of Mycobacterium avium and M. tuberculosis grown in Tween medium were loss of acid-fastness, increase in granulation, and at times, especially in highly bacteriostatic concentrations, shortening of the rods. When M. tuberculosis was grown in different media such as peptone, casein, beef extract, serum, albumin, amino acids, inorganic compounds, or glycerol, normal acid-fast cells were found only in those media which contained the more complex forms of nitrogen; otherwise, the damage to the cells by streptomycin
could not be neutralized by the presence of any of the
nutrients used.

Effect on Bacterial Metabolism

Effect on Amino Acid and Protein Metabolism

Geiger (1947), because of the lack of effect of streptomycin upon the oxidation of nitrogen-free carbon compounds by *E. coli*, directed his attention towards the oxidation of amino acids. He found that the rate of oxidation of most amino acids, including serine, threonine, alanine, leucine, histidine, arginine, lysine, and glutamic acid was small and unaffected by streptomycin. However, when alanine, leucine, serine, or glutamic acid was added to a cell suspension that had previously been permitted to oxidize fumarate, which is formed from the rapid deamination of aspartate, an immediate increase in oxygen uptake was observed in the absence of streptomycin, but not in its presence.

Table 13 summates the effect of streptomycin upon the oxidation of serine by *E. coli*. To obtain these results, 2 ml. of an *E. coli* cell suspension (0.051 mg. N/ml.) in Ringer phosphate solution was shaken for three hours with or without 0.2 ml. of 0.2 M fumarate solution in addition to the indicated concentration of streptomycin. Then 0.2 ml. of 0.2 M DL-serine was added and the oxygen uptake observed. These results suggested that an unidentified intermediate that had been formed in the course of the oxidation of fumarate by
TABLE 13

EFFECT OF STREPTOMYCIN UPON THE OXIDATION OF SERINE BY ESCHERICHIA coli

<table>
<thead>
<tr>
<th>Streptomycin (μg/ml)</th>
<th>Oxygen uptake/hr./mg. bacterial N</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serine alone (mm.)</td>
<td>Serine plus fumarate (mm.)</td>
</tr>
<tr>
<td>0</td>
<td>587</td>
<td>1450</td>
</tr>
<tr>
<td>0.5</td>
<td>627</td>
<td>1720</td>
</tr>
<tr>
<td>2.0</td>
<td>660</td>
<td>835</td>
</tr>
<tr>
<td>10.0</td>
<td>532</td>
<td>834</td>
</tr>
</tbody>
</table>
E. coli was a necessary part of the amino acid-metabolizing system of this organism, and that the metabolism of amino acids in the presence of this intermediate was prevented by streptomycin.

In order to determine the source of this intermediate, the oxidation of fumarate and other compounds, such as malate, succinate, oxaloacetate, glucose, lactate, pyruvate, and glycerol, was studied. Two ml. of E. coli suspension (0.063 mg. N/ml.) in Ringer phosphate solution containing cocarboxylase (0.1 ml., 0.1 mg./ml.) was shaken for three hours with 0.2 ml. of a 0.1 M solution of each of the above compounds; 0.2 ml. of 0.2 M DL-serine was then added and the oxidative metabolism observed. The results, as outlined in Table 14, show that the hypothetical intermediate was formed from the oxidation of malate, succinate, oxaloacetate, glucose, lactate, and glycerol as well as from fumarate, but not from pyruvate. In addition, it was found that the presence of phosphate or cocarboxylase aided in the formation of the intermediate from fumarate.

Another series of experiments was then performed to show whether the hypothetical intermediate was associated with the cells or with the suspending medium. The procedure was to shake a suspension of E. coli cells in air with fumarate and cocarboxylase for three hours, then to centrifuge and resuspend the cells in Ringer-phosphate solution and add serine. It was found that when streptomycin was present throughout
### TABLE 14

RATE OF OXIDATION OF SERINE IN THE PRESENCE OF VARIOUS CARBON COMPOUNDS

<table>
<thead>
<tr>
<th>Carbon Compounds</th>
<th>Oxygen Uptake/hr./mg, Bacterial N*&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Streptomycin absent</th>
<th>Streptomycin, 8Y/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mm.</td>
<td>mm.</td>
</tr>
<tr>
<td>Fumarate</td>
<td>1032</td>
<td>428</td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>1510</td>
<td>540</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>1670</td>
<td>667</td>
<td></td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>1080</td>
<td>357</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>2090</td>
<td>675</td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>1150</td>
<td>572</td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>198</td>
<td>198</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>1590</td>
<td>810</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Average for 3 hours, except for lactate, pyruvate, and glycerol, where the figures represent a 2-hour average.
the experiment, the cells oxidized serine much less rapidly than cells not subjected to streptomycin. The results were much the same if streptomycin was present only during the oxidation of fumarate and no more was added to the resuspended cells. These experiments suggest that the hypothetical intermediate needed for the rapid oxidation of amino acids by *E. coli* is located within the cells and not in the suspending medium.

To identify this intermediate, a study of the rate of oxidation of serine in Ringer-bicarbonate solution was made. Since there was no increase in the rate of oxidation of serine, the possibility that the intermediate was carbon dioxide was eliminated. Geiger suggests that the intermediate, since it is formed from lactate and glycerol, may be a $C_3$ compound, which is phosphorylated because of the necessity of phosphate for its formation.

Using the observations of Geiger as a starting point, Umbreit (1949) was able to trace the mode of action of streptomycin on *E. coli* somewhat further. He found that, as with serine, the oxidation of threonine was markedly increased by the previous oxidation of fumarate. However, if streptomycin was present during the oxidation of fumarate, little increase in the rate of threonine oxidation was observed. Therefore, this investigator proposed that streptomycin prevents the formation or activity of some substance derived from the oxidation of fumarate which is necessary for the oxidation of
threonine or its deamination product, \( \alpha \)-ketobutyrate.

It was, therefore, desirable to distinguish between the possibility that streptomycin prevented the formation of this substance, and the possibility that it prevented its action. He reasoned that, if fumarate were oxidized in the absence of streptomycin, the unknown substance should be formed. Furthermore, if the action of this substance was prevented by streptomycin, then the addition of the streptomycin with the threonine should be effective. On the other hand, if the formation of this intermediate was prevented by streptomycin, the latter should be active only when present during fumarate oxidation. The results indicated that streptomycin, to be really effective, must be present during fumarate oxidation. Therefore, streptomycin apparently acts by preventing the formation of the hypothetical intermediate rather than by interfering with its action once it is formed.

In an effort to identify this substance, experiments were done at a low phosphate level, at which the effects of streptomycin upon the respiration of fumarate itself could be observed. While it was apparent that there was some variation in the rate and extent of fumarate oxidation, there was also consistently less complete oxidation of fumarate in the streptomycin-treated cells. This investigator also observed that the oxygen uptake proceeded to at least 1 mole of \( O_2 \) per mole of fumarate at which time a marked streptomycin
Effect was evident. Since the utilization of 0.5 mole of \( \text{O}_2 \) per mole of fumarate would bring it to the oxidation state of pyruvate, there must be a utilization of about 0.5 mole of \( \text{O}_2 \) per mole of pyruvate before the streptomycin effect would be noted. Since this was confirmed, the general conclusion was drawn that streptomycin prevents the "terminal respiration" process in the susceptible bacteria. This was true of threonine (or the keto acid derived from it), in which a previous oxidation of fumarate was necessary before it could enter the terminal respiratory system, as well as for fumarate and pyruvate in which only a partial oxidation was observed when streptomycin was present.

Henry et al. (1949) found that there was a decrease in the total nitrogen content of \( \text{B. cereus} \) following bacteriostasis of the bacteria by exposure to streptomycin. Table 15 shows that the nitrogen content of the bacteria did not change appreciably in the control with or without added glucose, but when inhibition of multiplication occurred with streptomycin, the nitrogen content was somewhat lower. They postulate that this decrease in nitrogen content may be related in some way to the interference with amino acid metabolism by streptomycin that was reported by Geiger (1947).

Massart et al. (1947b) showed that the activity of streptomycin and acridines (and basic dyes in general) is due to a competition between streptomycin or acridines and physiological cations, more especially hydrogen ions, in
<table>
<thead>
<tr>
<th>Source of cells analyzed</th>
<th>µg SM/ml.</th>
<th>% Inhibition of multiplication</th>
<th>µg N/ml. T.U.</th>
<th>µg P/ml. T.U.</th>
<th>µg glucose/ml./T.U.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells at 0 hr.</td>
<td></td>
<td>2.1</td>
<td>.054</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>Control (no glucose)</td>
<td></td>
<td>2.0</td>
<td>.051</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>Control (0.4% glucose)</td>
<td></td>
<td>2.3</td>
<td>.044</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>SM &amp; glucose</td>
<td>.05</td>
<td>18</td>
<td>2.1</td>
<td>.034</td>
<td>0.43</td>
</tr>
<tr>
<td>&quot;</td>
<td>.10</td>
<td>88</td>
<td>1.8</td>
<td>.038</td>
<td>0.50</td>
</tr>
<tr>
<td>&quot;</td>
<td>.15</td>
<td>100</td>
<td>1.65</td>
<td>.030</td>
<td>0.53</td>
</tr>
<tr>
<td>&quot;</td>
<td>.50</td>
<td>100</td>
<td>1.7</td>
<td>.033</td>
<td>0.56</td>
</tr>
</tbody>
</table>

T.U. = turbidity unit  
SM = streptomycin
electro-adsorption complexes. This competition may be represented as follows:

\[(\text{NP}^x^-)(\text{xH}^+)=\text{electro-adsorption complex}\]

cation of streptomycin

where NP stands for nucleoproteins.

As a result of this competition, they found that streptomycin and acridines interfere with the metabolism of nucleoproteins by displacing such physiologically active ions as hydrogen from these electro-adsorption complexes.

Cohen (1947) used streptomycin and desoxyribonuclease to study the surface structure of the T₂ bacteriophage of E. coli. He found that streptomycin appears to react with phosphorylated nucleic acids to produce polymeric compounds, the size of which depended upon the combining ratios of the bivalent base (streptidine) to the multivalent nucleates. However, streptidine itself was found to be inactive in producing these lattices. Thus, the entire streptomycin molecule must be active in blocking or inhibiting the action of the enzyme desoxyribonuclease.

This work was confirmed by Krampitz and Werkman (1947) and Krampitz et al. (1947), who showed that streptomycin, like penicillin, inhibits the oxidation or dissimilation of the cellular ribonucleic acid and sodium ribonucleic acid, which is employed as a substrate for S. aureus and other bacteria.
Effect on Carbohydrate Metabolism

Henry et al. (1948), while working on the metabolism of resting cells of *S. aureus*, *B. cereus*, and *S. sonnei*, found that streptomycin inhibited the oxidation of certain carbohydrates and carbohydrate intermediates by susceptible strains of these organisms, but had no inhibitory effect on resistant strains. In order to show this, they studied the effects of streptomycin on the aerobic and anaerobic metabolism of these bacteria and on certain purified enzyme systems:

1. Aerobic Metabolism:

Colorimetric methods were used for the determination of the following substrates: glucose, pyruvate, lactate, ethanol, and glycerol, while acetate was determined by the steam distillation and titration method of Elliot et al. (1942). They showed that 1 µg. of streptomycin inhibited the oxidation of all the substrates used by the sensitive strain of *B. cereus*, while a similar concentration of streptomycin inhibited only the glycerol and lactate oxidation of susceptible strains of *S. aureus*, and all but the ethanol oxidation of susceptible strains of *S. sonnei*.

2. Anaerobic Metabolism:

1 µg of streptomycin per ml. produced inhibition of only two substrates, glucose and pyruvate.

3. Certain Purified Enzyme Systems:
Manometric techniques, using constant volume Warburg respirators, were used to measure rabbit blood catalase, yeast carboxylase, urease, carbonic anhydrase, succinoxidase, and cytochrome-cytochrome-oxidase. However, streptomycin, in very high concentrations (500 μg per ml.) did not inhibit these purified enzyme systems.

Therefore, since they found that the inhibition of metabolic functions by streptomycin seldom reached 100%, these experimenters postulated that streptomycin: (1) blocks some intermediate carbohydrate reaction, or (2) inhibits the functioning of an enzyme involved in carbohydrate metabolism by combining with it specifically, or (3) blocks the formation of some substance (enzyme? coenzyme? substrate?) essential for carbohydrate metabolism. Thus, they concluded that the observed inhibition of oxidative metabolism would be an indirect consequence of the primary inhibition.

Henry et al. (1949) used strains of S. sonnei to study the effect of streptomycin on the utilization of glucose and pyruvate by multiplying bacteria. The results are summarized in Table 16. In examining this table, it is obvious that the percentage of oxygen consumption, which compares the observed oxygen consumption with that calculated for complete oxidation of the substrate utilized to CO₂ and H₂O, did not change significantly. However, it is important to note that at the point of complete or nearly complete inhibition of multiplication the oxygen consumption is only partially inhibited.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>SM</th>
<th>% Inhibition of multiplication</th>
<th>% Inhibition of O₂ consumption</th>
<th>% Inhibition of Substrate used</th>
<th>% Recovery C as HAc from substrate used</th>
<th>% of theoretical O₂ consumption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>μg/ml.</td>
<td>T.U.</td>
<td>T.U.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>22</td>
<td>60</td>
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<td>70</td>
<td>70</td>
<td>40</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

T.U. = turbidity unit
SM = streptomycin
Therefore, they concluded, as did McCloy (1947) in studying narcotic inhibition of cellular activity, that, if the inhibition of oxidative metabolism is the primary cause of bacteriostasis, it would not be necessary that the total oxygen consumption be completely blocked in order to produce bacteriostasis. Thus, the oxidative metabolism of multiplying bacteria, as well as that of resting bacteria, shown by Henry et al. (1948), is inhibited by concentrations of streptomycin that produce bacteriostasis. In addition, they found that, coincident with the inhibition of multiplication and oxygen consumption, there is also inhibition of carbohydrate substrate utilization. These observations, along with changes in total phosphorus content and in total reducing substances following streptomycin bacteriostasis of B. cereus, leave little doubt that, together with streptomycin inhibition, there is interference in the carbohydrate metabolism of certain bacteria. As can be seen in Table 15, the phosphorus content in controls without glucose remained fairly constant, but with glucose added, the amount of phosphorus decreased. In addition, simultaneously with streptomycin inhibition of multiplication, there was a further decrease in cellular phosphorus. On the other hand, the reducing substances in the cells decreased in the controls without glucose in the medium—this probably represented utilization of carbohydrate reserves. However, in controls with glucose added, the reducing substance content increased, and, where there was streptomycin
inhibition, it increased still further.

**Effect on Fat Metabolism**

Oginsky et al. (1950) studied the influence of streptomycin on the fatty acid oxidation in *M. tuberculosis*, avian type. Cell suspensions of this organism were, therefore, allowed to oxidize the even-numbered saturated fatty acids from C₂ to C₁₈, in the presence and absence of streptomycin. The results indicate that the oxidation of the lower fatty acids (C₂ to C₁₀) was not affected by streptomycin, but that of lauric, palmitic, and stearic acids was partially inhibited. Therefore, it was thought possible that, in this organism, there might be two alternative mechanisms for the oxidation of fatty acids: one for chain length C₂ to C₁₀, unaffected by streptomycin, and another for C₁₂ to C₁₈, sensitive to streptomycin. Furthermore, streptomycin appears to inhibit only the oxidation of the breakdown products of the fatty acids, but not the oxidation of the fatty acid chain itself. Average streptomycin inhibition with two other fatty acids was 15% with tuberculostearic acid and 31% with phthioic acid.

These investigators also studied the effect of streptomycin on the oxidation of stearic acid by *E. coli*. The sensitive strain that was used oxidized stearic acid after a lag period of one hour; thereafter, the rate continued high for the duration of the experiment. In the presence of streptomycin, the lag period was the same, but the rate of
oxidation thereafter was lower than in the absence of streptomycin. They believed that the inhibition of stearate oxidation by streptomycin resulted from inhibition of the entrance of the products of fatty acid oxidation into the "citric acid (or tricarboxylic) cycle" as described below. Furthermore, the lag observed in the oxidation of stearate was thought to be due to the necessity of accumulating the proper intermediates for entrance into this "citric acid cycle" rather than to the formation of adaptive enzymes, especially since growth studies did not reveal adaptive enzyme formation.

**Effect on Other Metabolic Activities**

Oginsky et al. (1949) represented the terminal respiratory system in *E. coli* as follows:

\[
\begin{align*}
\text{Pyruvate} & \quad \text{Acetate} \\
\text{Oxalacetate} & \quad \text{Unknown condensation products} \\
\text{Malate} & \quad \text{Succinate} \\
\text{Fumarate} &
\end{align*}
\]

\[
\text{oxidized by a cyclic process resembling the citric acid cycle.}
\]

Umbreit (1949), in an investigation of the effect of streptomycin on the terminal respiratory process of *E. coli*, considered two possibilities: (1) streptomycin inhibits the further oxidation of pyruvate to the acetate, i.e. inhibits an "active acetate" or 2-carbon intermediate; (2)
streptomycin inhibits the oxalacetate-pyruvate condensation, thus preventing a variety of substances from entering the terminal respiratory system that resembles the citric acid cycle.

A search for the formation of "active acetate" was not experimentally successful. On the other hand, when fumarate was added to the bacterial cells, there was a lag period of about 90 minutes before the maximum rate of oxidation was observed. During this period there was a gradually increasing rate of oxygen uptake, so that by the time the maximum rate had been reached, 0.5 mole of O₂ per mole of fumarate had been used. Since this would be sufficient to oxidize the fumarate to the oxalacetate stage, one may presume that the rate of oxidation thereafter was controlled by the oxalacetate and pyruvate (its decarboxylation product) condensations. Furthermore, since the addition of pyruvate at 3 hours had no effect, the rate-controlling factor was probably oxalacetate.

These results point definitely to the second hypothesis, i.e. that the net result of streptomycin inhibition is to prevent pyruvate (and evidently other keto acids) from entering the terminal respiration system by way of oxalacetate condensation.

Further evidence for this conclusion was presented by Oginsky et al. (1949). These investigators compared the activity of streptomycin in "old" and "young" suspensions of E. coli, especially with respect to oxalacetate oxidation,
since the fresh suspensions, unlike the older ones, had an active oxalacetate decarboxylase, which rapidly converted added oxalacetate to pyruvate.

Fresh cell suspensions initially oxidized oxalacetate at a slower rate than pyruvate. Without streptomycin, the rate of oxidation gradually increased; with streptomycin, it remained constant. This data was interpreted as follows: since oxalacetate cannot be oxidized as such, it must be decarboxylated and the resulting pyruvate oxidized, or it may condense with pyruvate to enter the "citric acid cycle". In the latter case, the oxygen uptake would be due to oxidation of products successively formed during the cycle, after the initial condensation reaction. However, such a condensation requires that pyruvate be present, a condition that would result only after a time sufficient to enable adequate quantities of pyruvate to result from the decarboxylation of oxalacetate. Therefore, the increasing rate of oxidation, which was inhibited by streptomycin, would be due to the occurrence of the oxalacetate-pyruvate condensation reaction, and the subsequent cyclic process.

With regard to the older cell suspensions, these men noted that pyruvate was oxidized normally, but oxalacetate was oxidized very slowly, presumably because it could not be decarboxylated. Therefore, when the product of decarboxylation was used, i.e. a mixture of oxalacetate and pyruvate, this condensation reaction occurred; furthermore, it was inhibited
by streptomycin. All these results tend to support the supposition that streptomycin acts by inhibiting the oxalacetate-pyruvate condensation.

According to Agnew et al. (1947), one of the properties of pathogenic staphylococci is the capacity to induce, by means of the enzyme staphylocoagulase, the formation of a fibrin clot when the organisms are added to citrated human plasma. Therefore, in studies on the inhibitory effect of streptomycin upon the action of staphylocoagulase, they noted that streptomycin markedly inhibited the action of staphylocoagulase, even in the presence of viable cells.

Two procedures were followed in detecting the effect of streptomycin on the action of staphylocoagulase. In procedure A, the coagulase and viability tests were set up immediately after the antibiotic and organisms had been mixed. In procedure B, mixtures of antibiotic and organisms were incubated for twenty-four hours at 37°C before carrying out the coagulase and viability tests. Although the use of procedure B delayed the clotting effect of staphylocoagulase longer than that obtained with procedure A, this retardation was not associated with an increased destruction of organisms. In conclusion, they say that, even though inhibition by streptomycin occurred in the presence of viable cells, this does not necessarily imply that streptomycin acts directly upon staphylocoagulase or blocks the reaction between staphylocoagulase and plasma.
Hirsch and Dosdogru (1947) determined the effect of streptomycin against the staphylococci by continuous measurement of the oxygen consumption of resting and proliferating cultures. They found that the addition of streptomycin does not harm the respiratory enzymes of the cocci in lactic acid-phosphate buffer, with the result that there is no change in respiration. However, the addition of 10μ to 500 μ/ml. streptomycin was found to upset the proliferation of staphylococci in nutrient solutions containing peptone and yeast extracts. This effect does not appear directly after the addition of streptomycin, but after a latency period, the length of which is shortened by an increasing amount of streptomycin. Consequently, the respiratory quotients decline slowly, like the respiratory quotients of resting staphylococci.

Schuler (1947), in studying the mode of action of six different antibiotics, found that streptomycin and penicillin act on the respiration of E. coli and on the staphylococci in the same manner. Both antibiotics show a latency period and an active inhibitory period. On the other hand, tytothricin, gramicidin, gliotoxin, and clavacin do not act in this manner.

Henry et al. (1948), in studying the effect of streptomycin upon the endogenous respiration of S. aureus and B. cereus, found that, although streptomycin in concentrations up to 2000 μg per ml. had no effect on the endogenous oxygen
consumption of S. aureus, the metabolism of the susceptible strain of B. cereus was altered. This was proven by comparing the respiratory quotients of the susceptible strains of B. cereus in the absence (R.Q. = 1) and presence (R.Q. = 1.4) of 10 μg streptomycin per ml.

Schoenbach and Chandler (1947), while studying the interrelationships that are present when bactericidal tests are performed with a strain of S. aureus in the presence of streptomycin, reported that the chemotherapeutic action of streptomycin was to inhibit bacterial multiplication or growth. They reached this conclusion after rejecting two other alternatives, i.e. streptomycin inhibition of growth of a resistant strain of S. aureus in the presence of whole blood or serum was due to (a) one of the components of rabbit serum that was used, or (b) the stimulation of phagocytosis for twenty-four hours, after which the leucocytes died and growth reappeared.

The first theory was rejected because no difference in inactivation of S. aureus was noted when normal rabbit serum, to which streptomycin had been added, was inoculated with bacteria at two different times. In one set of experiments, the normal rabbit serum was inoculated immediately and incubated for twenty-four hours. In a duplicate set, the normal rabbit serum was incubated for twenty-four hours, after which it was inoculated with organisms and again incubated for twenty-four hours.
The second possibility was disproven when it was found that, in the presence of streptomycin, the phagocytic activity of the white blood cells was depressed in every case. These experimenters suggest that, under normal conditions, two alternative growth mechanisms are available to the normal organism, such that streptomycin blocks one metabolic pathway, and prevents the other one from developing sufficiently to permit normal growth.

Welch et al. (1946) noted in some preliminary work that, while relatively high concentrations of streptothricin did not interfere with the ability of S. aureus to reduce nitrate to nitrite, somewhat lower concentrations caused complete inhibition of nitrate reduction. Therefore, they decided to see if this same type of phenomenon could be demonstrated if the organisms were incubated in vitro in the presence of streptomycin. Serial dilutions of streptomycin were made in broth and an equal quantity of a 1:100 dilution of a broth culture of E. typhosa was added. After twenty-four hours, 0.01 ml. of this mixture was transferred from each tube in the series in which there was no visible growth to a sterile Petri dish, and melted agar was added to form a pour plate. In all instances, the last tube to show inhibition contained very few viable organisms, while plates made from the one or two tubes preceding the last tube showing inhibition in the series, although containing larger amounts of streptomycin, contained large numbers of viable organisms.
Then, to determine whether this phenomenon of apparent "stimulation" could be elicited in vivo, an experiment was designed in which *E. typhosa* was used as an infective agent in white mice, and increasing concentrations of streptomycin were employed to protect, or to elicit the "stimulation" phenomenon. The results show that one-half ml. amounts of streptomycin solutions containing 0.05, 0.125, 0.5, and 1.0 microgram, when injected intraperitoneally, rather than protecting an increasing number of mice as the concentration of streptomycin was increased, actually resulted in a marked increase in the percentage of deaths over the controls which did not receive streptomycin.

In order to explain this phenomenon of apparent "stimulation" of a pathogen by streptomycin, they suggested two possibilities: (1) At certain concentration levels in the animal body, the stimulation in growth in size of the infecting organism, which is demonstrable microscopically, is carried over into the mechanism of cell division as well. Thus, the organism not only increases in size, but also in the number of divisions occurring in a given time interval. (2) If streptomycin only acts on dividing cells, cell division may be retarded to such a degree by the antibiotic that fission fails to occur until after the antibiotic has disappeared from the animal body. This second idea has been refuted by Henry et al. (1948), when they showed that streptomycin acts, not only on dividing or multiplying cells, but
on resting cells as well. 

Benham (1947) reported an apparent "stimulation" by streptomycin of the endogenous respiration of *E. typhosa*. When streptomycin, sufficient to make a final concentration of 1,000 units per ml., was added to a system in which endogenous respiration was proceeding at 37°C in a phosphate buffer of pH 7.4, an immediate and rather marked increase in the rate of oxygen uptake occurred. After two hours, the rate of oxygen uptake decreased until, at six hours, it was less than that of the controls. The addition of glucose in a concentration of 0.01% to a similar system also produced an increased rate of oxygen uptake, while a combination of glucose and streptomycin (1,000 units/ml.) at the same time to a suspension of *E. typhosa* produced similar results. The evidence indicates that the increase in oxygen uptake in the presence of streptomycin is not explainable on the grounds that the bacteria were bringing about the oxidation of streptomycin or impurities in it. It appears that the utilization of carbohydrate substrate is more complete and more rapid when streptomycin is present than when it is absent. Thus, Benham failed to obtain streptomycin inhibition of the oxidation of a carbohydrate substrate by *E. typhosa*.

Bernheim and Fitzgerald (1947) observed that streptomycin prevents the oxidation of benzoic acid by *M. tuberculosis* 607. More specifically, they found that 10⁻⁷ of streptomycin completely inhibited the oxidation of 1.0 mg. of benzoic acid.
by the normal strain of *M. tuberculosis*, whereas 100\% of streptomycin had no effect on the oxidation by the resistant strain of the same organism. Since the oxidation of pyruvic acid by the normal strain, as well as its oxygen uptake without added substrate, was not affected by 100\% of streptomycin, they concluded that the inhibition of benzoic acid by streptomycin was fairly specific. However, when it was found that the increased oxygen uptake by the virulent strain in the presence of benzoic acid was not affected by 300\% of streptomycin, they postulated that other mechanisms must be inhibited in this pathogenic strain.

Fitzgerald and Bernheim (1947), having found that the growth (but not the oxidative metabolism) of pathogenic mycobacteria which do not oxidize benzoic acid and non-pathogenic mycobacteria which oxidize benzoic acid is equally inhibited by streptomycin, postulated that there must be some general reaction which is inhibited by streptomycin, and that the inhibition of oxidative reactions is secondary. In their work, they showed that the oxidation of benzoic acid by most non-pathogenic mycobacteria is very sensitive to streptomycin. For example, 10. \mu g of streptomycin in 2.0 ml. of benzoic acid caused an appreciable inhibition, while 10.0 \mu g of 2.0 ml. caused complete inhibition. Then, in order to determine whether the oxidation of benzoic acid was the reaction most sensitive to streptomycin, various possible substrates, as shown in Table 17, were added to suspensions of the bacteria.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Benzoic Acid</th>
<th>Salicylic Acid</th>
<th>p-Hydroxy-Benzoic Acid</th>
<th>Trehalose</th>
<th>Fructose</th>
<th>Glucose</th>
<th>Mucic Acid</th>
<th>Acetic Acid</th>
<th>Palmitic Acid</th>
<th>Mannitol</th>
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<tr>
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<td>-X</td>
<td>/C</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>/</td>
<td>-</td>
<td>-</td>
<td>/</td>
<td>/</td>
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<tr>
<td>Mycobacterium BCG</td>
<td>/C</td>
<td>-</td>
<td>-</td>
<td>/</td>
<td>/</td>
<td>-C</td>
<td>/</td>
<td>/</td>
<td>/</td>
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<tr>
<td>Organism</td>
<td>Benzoic Acid</td>
<td>Salicylic Acid</td>
<td>p-Hydroxy-Benzoic Acid</td>
<td>Trehalose</td>
<td>Fructose</td>
<td>Glucose</td>
<td>Mucic Acid</td>
<td>Acetic Acid</td>
<td>Palmitic Acid</td>
<td>Mannitol</td>
</tr>
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</tr>
<tr>
<td>Mycobacterium phlei</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mycobacterium H37</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td></td>
<td>X</td>
<td>X</td>
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<tr>
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<td>C</td>
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<td></td>
<td>C</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
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</table>

/ = increased O uptake
- = no increased O uptake
C = utilized as sole C source
X = not utilized as sole C source
In all cases, streptomycin inhibited the oxidation of benzoic acid to a greater extent than any other compound tested.

The next problem that was undertaken was to learn whether the latent period - before the inhibition of the oxidation of benzoic acid takes effect - was due to a slow penetration of streptomycin into the bacterial cell, or to the fact that the antibiotic was not inhibiting the first step in the oxidation of benzoic acid, but some intermediate step. Streptomycin was added to the bacterial suspension at different intervals during the oxidation of benzoic acid, and the time required for the attainment of a given percentage of inhibition was measured. Since the time was the same whether the streptomycin was added before the benzoic acid or halfway through the oxidation process, the latent period was due to the time necessary for the drug to penetrate to its site of action. In addition, this shows that it is the oxidation of benzoic acid that is inhibited; however, the possibility still remains that the oxidation of intermediates is also affected.

As a result of increased interest in the ability of drugs to compete with essential metabolites, these investigators attempted to determine the effect of different concentrations of benzoic acid on inhibition by streptomycin, in order to see whether the two substances (substrate and inhibitor) are competing for the oxidase enzyme. The following percentages of inhibition were obtained when 1.0 and
2.0 mg. of benzoic acid, respectively, were added to a constant amount of streptomycin (5.0 μg/ml.):

1. 38% and 25% for M. avium
2. 25% and 12% for M. BCG

Since, as the oxidation proceeds, the percentages of inhibition increase and become the same for the two concentrations of benzoic acid, the indication is that streptomycin and benzoic acid compete for the enzyme (oxidase).

Fitzgerald and Bernheim (1948), in some preliminary work, found that organisms, such as M. tuberculosis, when grown on a normal medium, showed a latency period of 30-60 minutes before benzoic acid was oxidized, whereas those organisms that were grown in benzoic acid subsequently oxidized benzoic acid without this latent period, so that much more streptomycin was necessary to inhibit this oxidation. Therefore, they believed that this streptomycin insensitivity was due to the presence of more benzoic acid oxidase in the organisms grown in, and therefore adapted to, benzoic acid.

Stanier (1947), by use of the technique of simultaneous adaptation, showed that benzoic acid and such related compounds as phenylacetic acid, dl-mandelic acid, benzaldehyde, and para-hydroxybenzoic acid, are oxidized by adaptive enzymes in Pseudomonas fluorescens. This adaptation occurred one hour after the compounds had been added to non-growing cell suspensions in Warburg respirators, i.e. it required
about an hour for the formation of the adaptive enzyme.

Fitzgerald and Bernheim (1948), in observing the work of Stanier, felt that perhaps a similar rapid adaptation occurred in mycobacteria. Therefore, they postulated that the inhibition of benzoic acid oxidation by streptomycin may not be due primarily to the inhibition of the benzoic acid oxidase, but to the inhibition of the formation of the enzyme. In order to test this hypothesis, 40 mg % benzoic acid was added to a medium containing the bacteria, M. lacticola. After forty-eight hours' growth at room temperature, the bacteria were centrifuged and washed, and a standard suspension was made. Aliquots were placed in Warburg vessels and the oxygen uptake was measured in 0.2 M phosphate buffer pH 6.7, in the presence of benzoic acid with and without different amounts of streptomycin. The results, which compare the normal medium to the medium containing 40 mg % benzoic acid, may be summarized as follows:

A. In normal medium:

1. 50 μg SM/liter inhibits the oxidation of benzoic acid by the organisms 44%.
2. 100 μg SM/liter inhibits the oxidation of benzoic acid by the organisms 82%.
3. 200 μg SM/liter inhibits the oxidation of benzoic acid by the organisms 100%.

B. In 40 mg % benzoic acid medium:

1. 50 μg SM/liter inhibits the oxidation
of benzoic acid by the organisms 25%.
2. 100 μg SM/liter inhibits the oxidation of benzoic acid by the organisms 30%.
3. 200 μg SM/liter inhibits the oxidation of benzoic acid by the organisms 33%.

These results suggest that the enzyme was relatively insensitive or unaffected by streptomycin, and that the inhibition by streptomycin of the oxidation of the benzoic acid is caused by the suppression of enzyme formation. Therefore, the previously published report by Fitzgerald and Bernheim (1947), which showed that streptomycin caused less inhibition in the presence of 2.0 mg. of benzoic acid than in the presence of 1.0 mg. could be interpreted to mean that 2.0 mg. caused the production of more enzyme.

Influence of Culture Medium (especially pH) on Streptomycin Activity

While studying the mode of action of streptomycin, it is necessary to consider an important environmental factor which may greatly influence antibacterial activity, i.e. the composition of the culture medium.

Foster and Woodruff (1943b) noted that the presence of phosphates, some other salts, and sugars in test media decreased the activity of streptothricin. After they had observed that raising and lowering the pH of the medium increased and decreased, respectively, the streptothricin activity, they concluded that phosphates and sugars caused
the observed interference through effects on pH. In order to account for the increased activity at a higher pH, they reasoned that only the undissociated free base of streptothricin was active.

Similar observations with regard to streptomycin were presented by Waksman et al. (1944) and Waksman and Schatz (1945) who observed the effects of pH and glucose on the bacteriostatic activity of streptomycin and streptothricin. They found that both antibiotics are similarly sensitive to an increasing acidity with the result that they are considerably more effective at a higher than at a lower pH. These results are summarized in Table 18.

Other investigators have also demonstrated that the action of streptomycin varies markedly with change in pH. For example, Loo et al. (1945) found that the size of the zone of inhibition that resulted with a solution of constant streptomycin concentration increased markedly by raising the pH of the solution.

Wolinsky and Steenken (1946), in studying the effect of variation of pH on streptomycin activity, adjusted tubes of F.D.A. broth, consisting of peptone, Bacto beef extract, sodium chloride, and distilled water, to the following pH's: 5.2, 6.6, 7.2, and 7.7. To these tubes enough streptomycin was added to give concentrations of 0.4 to 200 units per cc. Each tube was then inoculated with 0.1 cc. of a twelve-hour broth culture of staphylococcus, diluted ten times. Table 19
TABLE 18

EFFECT OF pH AND GLUCOSE ON THE BACTERIOSTATIC ACTIVITY
OF STREPTOMYCIN AND STREPTOTHRICIN

Activity in units per 1 ml. *

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Streptothricin 1 ml. = 5 mg.</th>
<th>Streptomycin 1 ml. = 100 mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH**</td>
<td>Reaction Effect</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1,790</td>
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<tr>
<td>8</td>
<td>2,200</td>
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<tr>
<td>7</td>
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<td>6</td>
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<td>700</td>
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<td>5</td>
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</table>

<table>
<thead>
<tr>
<th>Glucose***</th>
<th>Glucose Effect</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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<tr>
<td>2</td>
<td>1,440</td>
</tr>
<tr>
<td>10</td>
<td>1,480</td>
</tr>
</tbody>
</table>

* A unit is the amount of material that will inhibit the growth of E. coli in 1 ml. of medium.

** Figures show the final pH.

*** Glucose, mg. per plate.
TABLE 19

CONCENTRATIONS OF STREPTOMYCN NECESSARY FOR COMPLETE AND PARTIAL INHIBITION OF GROWTH AT DIFFERENT pH LEVELS. INCUBATION 24 HOURS

<table>
<thead>
<tr>
<th>pH</th>
<th>Complete Inhibition</th>
<th>Partial Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.7</td>
<td>1.6</td>
<td>0.8</td>
</tr>
<tr>
<td>7.2</td>
<td>6.25</td>
<td>1.6</td>
</tr>
<tr>
<td>6.6</td>
<td>6.25</td>
<td>3.2</td>
</tr>
<tr>
<td>5.9</td>
<td>50.00</td>
<td>25.0</td>
</tr>
<tr>
<td>5.2</td>
<td>100.00</td>
<td>40.0</td>
</tr>
</tbody>
</table>

Inoculum 0.1 cc. 12-hr. broth culture diluted 10 times.
indicates that the inhibitory efficiency of streptomycin in broth diminishes with increasing acidity.

Then, in order to determine whether the drug is actually destroyed in acid solution, they prepared tubes of broth of a pH of 3.5, 6.4, and 7.2 so that they contained ten units of streptomycin per cc. After the tubes were incubated for two hours, they were quickly adjusted to pH 7.8 with 0.1N NaOH, and streptomycin assays were made. All the tubes were found to contain about ten units of streptomycin per cc., thus indicating that there had been no destruction of the drug.

An interesting observation was brought out by Berkman et al. (1947) when they reported that the effect of low pH (pH 3.0 for 24 hours) on streptomycin activity is reversible. This was indicated by the fact that such solutions of streptomycin regained their activity at pH 7.8.

Brown and Young (1947) studied the effect of variation of the pH on the end-point concentration of streptomycin. It was found that the pH of the broth used for the routine tests fell from pH 7.6 to 7.4 on re-autoclaving, and the variation in activity from pH 7.2 to 7.4 and from 7.4 to 7.6 was more than 20%. This signified that, for each decrease of 0.1 unit of pH between 7.6 and 7.2, the streptomycin activity was decreased by about 10%.

Abraham and Duthie (1946) suggested the possibility of bacterial (staphylococcal) cell surface changes accompanying alterations in the pH. By means of serial dilution and
cylinder plate tests, they found that the pH was a critical factor in determining the fate of the bacterial cells when streptomycin was at the borderline of its bactericidal concentration. Five units per cc. of streptomycin in an inoculum of 300 bacteria per cc. appeared to exert a progressive killing effect at both pH 7.0 and 8.0, while at pH 6.1 the same amount of drug had no bactericidal effect and the culture was fully grown in about eighteen hours.

In order to account for the increased activity of streptomycin as the pH of the medium is raised, they presented two different hypotheses. One theory was that the reaction effect may be due to the fact that the free base, possibly because it is able to penetrate the lipoid material of the cell, is more active than its cation. For example, bases such as streptomycin, which have pK values of approximately 9.0, are largely in the cationic form between pH 6 and 8, so that in this range the concentration of free base in solution increases nearly ten times with a rise in pH of 1 unit. Secondly, assuming that the cation is mainly responsible for the activity of the base, they postulated that the reaction effect may be due to competition between hydrogen ions and base cations for centers on the cell surface. Hence, at a lower pH the active part of the streptomycin molecule is neutralized by the acid resulting in a decrease in antibiotic activity.
In reporting the interfering action of sugars on streptomycin activity, Waksman and Schatz (1945) observed that such effects were probably caused by pH changes in the medium, but they also mentioned the possibility of the reducing role played by sugars.

Lenert and Hobby (1947), in order to demonstrate the effect of the medium on the sensitivity of an organism to streptomycin, compared the inhibition of streptomycin activity by peptone with the non-inhibitory effect that was obtained in beef infusion broth. They postulated that the inhibitory action of the peptone broth on the antibacterial action of streptomycin may be due, in part, to an alteration in the oxidation-reduction potential of the medium. The non-inhibitory effect of the beef infusion broth was accounted for on the basis of the presence of one or more substances which counteracted the effect of the peptone on the action of streptomycin.

Van Dolah and Christenson (1947) treated streptomycin in aqueous solutions with a large number of agents, both oxidizing and reducing, in an attempt to study inactivation in terms of such reactions. In direct conflict with Lenert and Hobby (1947), they found that the inactivation of streptomycin could not logically be ascribed to an oxidizing nor reducing mechanism, but resulted from a specific reaction or an interference mechanism.

This latter idea was confirmed by Geiger et al. (1946),
who concluded that the effects of sugars were caused by pH changes, since glucose was found to have no effect on streptomycin in the presence of a glycine buffer. However, this conclusion was questioned by Donovick and Rake (1946) when they found that increased glucose concentrations, under the conditions of the test, did not lead to a lower pH, but did decrease the activity of the antibiotic.

Studies by Wallace et al. (1945) and Rhymer and Wallace (1947) have indicated that the influence of the composition of the medium on the antibacterial activity of streptomycin is due to something that can be extracted from the culture media. In their work, the following culture media were prepared: (a) Nutrient broth, (b) Nutrient broth diluted with an equal amount of water, and (c) Brain heart infusion. Streptomycin was added in varying amounts to tubes containing 15 ml. of each of these media, and *E. typhosa* and *S. aureus* were added. The cultures of both groups of bacteria showed active growth in brain heart infusion, while those in nutrient broth and dilute nutrient broth rapidly decreased in numbers. This signified that brain tissue contains large amounts of an active substance which Rhymer et al. (1947) suggested to be lipositol. Therefore, it was apparent that there was some active substance in the brain heart infusion, but not in the nutrient broths, which interfered in some way with the action of streptomycin.
Origin of Bacterial Resistance to Streptomycin

As in the case of penicillin, there are two theories which attempt to explain the origin of bacterial resistance to streptomycin: (1) metabolic adaptation theory, and/or (2) mutation (genetic) theory.

Metabolic Adaptation Theory.

Sureau et al. (1948) found that filtrates of cultures of *Bacillus pyocyaneus* and certain enterococci are capable, even at small concentrations, of inhibiting streptomycin because of the production of the enzyme streptomycinase by these bacteria. They defined this enzyme by the following characteristics: (a) it has a definite specificity to inactivate streptomycin, but will not inhibit penicillin; (b) it has no definite antibiotic action of its own; and (c) it functions like other enzymes. There has been no other claim to the production of such an enzyme by other authors.

In order to refute this theory, Graessle and Frost (1946) showed that none of their resistant staphylococcal strains destroyed streptomycin. Furthermore, Seligmann and Wassermann (1947) devised experiments to decide whether resistant strains of *Salmonella*, growing in streptomycin solutions, destroyed all or part of the streptomycin during their growth stage. They found that acquired streptomycin resistance was not connected in any way with an increased destructive power of the micro-organisms.

Further work by these latter investigators on the develop-
ment of resistance by certain chromogenic bacteria and various strains of Salmonella revealed distinct and rapid alterations of the metabolic activities of all resistant strains. For example, their growth rate was slower, their reducing intensity was lowered, and their enzymatic activities, involving carbohydrate fermentation and $H_2S$ production, were changed. In addition, pigment production of B. pyocyaneus was impaired, and loss of virulence was observed in Salmonella strains. The regularity of the effect that was obtained was impressive, since all original sensitive strains retained their original cultural and biological characteristics, and all resistant strains showed the marked alterations.

These authors suggested that, correlated with other enzymatic losses, a streptomycin "receptor" of the bacterial cell may become lost or weakened, so that the resistant strains have an altered "lock" the "streptomycin key" no longer fits. Thus, they proposed that streptomycin produced a physiological response of the bacterial cell against the damaging drug with the result that the organisms either died (sensitive strain) or they survived (resistant strain) with a damaged enzymatic system.

During a study of acquired resistance to streptomycin made by Stubblefield (1947), a culture of E. coli was found to give rise to a round-cell resistant variant which could be maintained in culture. Although the variant and the parent strain gave identical biochemical reactions, antigenically
the two strains were not alike. This was revealed by adsorption studies, which demonstrated the presence of an antigen in the round cells which was not present in the original rod cells.

A study by Gezon and Fasan (1949) has further revealed that there were both antigenic and enzyme system changes in beta hemolytic streptococci, made resistant to penicillin and streptomycin. Representative members of group A and C streptococci were studied for alterations in: (1) streptomycin; (2) streptokinase; (3) proteinase; and (4) ribonuclease. Table 20 correlates changes in the bacterial enzyme systems of organisms with induced antibiotic resistance. It may be observed that some group A streptococci, after acquiring either penicillin or streptomycin resistance, may show reduction in enzymatic capacity or lose some antigenicity. On the other hand, the acquisition of aureomycin or bacitracin resistance caused little change in the activity of these group A streptococci from that of the parent strain.

Although the parent group C strains that were studied produced neither proteinase nor ribonuclease, the three strains resistant to penicillin and streptomycin tended to show a significant decrease in activity from that of the control strains in both streptolysin S and streptokinase production. However, the bacitracin- and aureomycin-resistant organisms maintained their activities at the same level as the parent strains. These authors believed that the mechanism of
<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Antibiotic</th>
<th>Streptolysin S</th>
<th>Streptokinase</th>
<th>Proteinate</th>
<th>Ribonuclease</th>
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<tr>
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<td>Aureomycin</td>
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**/** = Activity of the parent and control strains

# = Eight-fold or greater decrease from that of parent and control strains
understanding this acquired antibiotic resistance with the co-existing streptococcal behavioral changes would come from typing the parent and resistant strains.

Smith et al. (1949) have shown that the parent strain of E. coli and the derived resistant variants differed metabolically in three ways. The sensitive parent strains were able to benefit by the presence of air during growth, were able to oxidize rapidly the intermediates of the "citric acid cycle", and possessed an enzyme causing the oxalacetate-pyruvate condensation. The resistant strains, on the other hand, have lost these properties, and have developed an alternative pathway capable of forming substances vital to the cell, which result from the condensation in the sensitive strain. Therefore, it appeared that the ability of the resistant strains to grow in the presence of streptomycin depended upon the development of unknown reactions which permitted the bacterial cell to dispense with the oxalacetate-pyruvate condensation.

Klimek et al. (1948), in studying the development of resistance by S. aureus to streptomycin, found that resistance developed at a rapid rate. During the course of seven sub-culturings in broth containing streptomycin, the resistance of the culture increased from 0.5 micrograms to 1 mg. per ml. After twelve transfers the organisms grew well in a broth containing 4 mg. per ml. of streptomycin. Although no significant morphological changes were observed, they noticed
that streptomycin modified the characteristic fermentation reactions of *S. aureus*.

Similar work by Graessle and Frost (1946) revealed that some streptomycin-fast staphylococcal strains showed an alteration in metabolic activities, such as a loss of pigment production and a reduction in the rate of carbohydrate fermentation.

Further evidence in favor of the metabolic adaptation theory was presented by Hall and Spink (1947), who found changes in colony characteristics in a strain of *Brucella abortus* which had developed marked resistance to the action of streptomycin. Two distinct colony forms were noted: (1) A large colony type which grew in as much as 50,000 units of streptomycin per ml. These organisms, however, did not ferment any of the sugars normally fermented by *B. abortus*. (2) A small colony was much more numerous and was composed of organisms showing the same fermentation and serological reactions as the sensitive strain, although it could grow in the presence of 7,500 ug. per ml. of streptomycin. Since these organisms died out readily unless heavy inocula were employed, a decrease in the production of some essential metabolite was suggested.

Although Murray et al. (1946) found that gram-negative bacilli, which were made resistant by exposure to streptomycin in broth, showed marked pleomorphism and, in some instances,
underwent changes in their biochemical reactions, there was less pleomorphism and no changes in biochemical reactions when resistance developed as a result of exposure to the agent on surface plates. In further opposition to the metabolic adaptation theory, they observed that resistant organisms obtained from the patient during or after treatment showed no morphological or cultural differences from the original sensitive strains isolated from the same patient.

In conclusion, it is interesting to note the observation of Welch et al. (1949) that resistance of *Salmonella typhosa* to streptomycin was induced as a result of the action between the antibiotic and the bacteria. More specifically, they showed that the anionic portion of the streptomycin molecule markedly affected the ability of the organism to develop resistance to this drug. In order to prove this hypothesis, they tested the ability of both fatty acid salts (caprylate, undecylenate, and propionate) and commercially available salts of streptomycin in preventing an increase in resistance of a strain of *S. typhosa*. These investigators observed that this organism increased in resistance to streptomycin CaCl\(_2\) to a far greater extent than to the fatty acid salts that were used. In addition, when this strain was made resistant to high concentrations of CaCl\(_2\) complex salt of streptomycin, it had relatively low resistance to the fatty acid salts of this same antibiotic. Conversely, *S. typhosa*, having been made resistant to relatively low concentrations of the fatty
acid salts of streptomycin, had a relatively high resistance to the commercially available salts. These results indicated that the anionic portion of the molecule influenced the activity of streptomycin against certain strains of bacteria, and that resistant cells arose, not before contact, but as a result of contact with the antibiotic.

**Mutation (genetic) Theory**

Demerec (1948), by a similar method and rationale as was used for penicillin, was able to establish the concept that resistance to streptomycin originates by mutation, independent of the action of the antibiotic. From a single culture of bacteria, small inocula (50-300 bacteria) were taken and used to start 21 or more independent broth cultures, which were incubated for twenty-four hours, or until growth had reached the saturation point. During this period of incubation, the number of bacteria increased to about $2 \times 10^6$ per ml.

Then, from the same culture that served as the source of the inocula, ten samples of bacteria of the same size as the inocula were placed on a culture medium containing the same concentrations as were used in the tests. This was done in order to determine if any resistant bacteria were present in these samples. Since no resistant organisms were found, it followed that all resistant bacteria occurring in fully-grown cultures had necessarily originated in these colonies during the period when the number of bacteria increased from
50-300 to about $2 \times 10^8$ per ml.

Next, from 20 of the broth cultures, samples of 0.1 ml. were taken and plated on Petri dishes containing the same concentration of streptomycin ($5$ units per ml. of medium). From the twenty-first tube, fifteen 1 ml. samples were plated. Thus, two sets of plates were obtained, one set of twenty in which each plate had bacteria from a different culture, and another set of fifteen, all having bacteria from the same culture. These plates were then incubated for twenty-four hours, or for a longer period if the growth of colonies was slow, after which the number of colonies on each plate was determined. These colonies represented resistant bacteria that had been present in the sample plated.

Since the experimental conditions were similar on both sets of plates, like numbers of bacteria ($\approx 2 \times 10^8$) having been plated onto nutrient agar containing identical concentrations of streptomycin, this investigator reasoned as follows: if resistance was induced through interaction between the bacteria and streptomycin, approximately similar numbers of resistant bacteria would be obtained on all the plates, regardless of the origin of the bacterial samples. However, if the origin of resistance was mutational, similar numbers of resistant colonies would be obtained only among the platings from the same culture, since these represented repeated tests of the same mixture of resistant and sensitive
bacteria. Among the samples from separate cultures, if
mutations occurred at random, there would be a large number
of resistant colonies from cultures in which mutation happened
to occur early in the growth of the cultures, and a small
number of resistant colonies from cultures in which the
mutation happened to occur late, provided the growth rate of
the resistant bacteria was not appreciably different from
that of normal ones. Therefore, if resistance originated by
mutation, the variation in numbers of resistant organisms
would be much greater between samples taken from separate
cultures than between samples taken from the same culture.

Table 21 shows the result of such an experiment, per-
formed with E. coli and streptomycin. It is easily evident
that the variation in the number of resistant colonies was
considerably greater among platings from independent (separate)
cultures than among platings from a single culture. These
results favor the idea that resistance to certain concentra-
tions of streptomycin originated through mutations, and that resis-
tant bacteria may be found in any large population, the
proportion depending on the mutation rates.

This investigator showed that the building up of resis-
tance to streptomycin, unlike penicillin, did not follow a
definite pattern, but showed a considerable degree of vari-
bility. For example, among first-step resistant strains,
i.e. strains isolated from colonies of the original strain
that survived sublethal doses of streptomycin, there were
<table>
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<tr>
<th>Culture no.</th>
<th>No. of resistant bacteria</th>
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<td>101</td>
<td>20</td>
<td>164.4</td>
<td>10</td>
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</table>

Average: 105.9
Variance: 2913.9
Chi-square: 550.3
P much less than: 0.001

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<th>Culture no.</th>
<th>No. of resistant bacteria</th>
<th>Culture no.</th>
<th>No. of resistant bacteria</th>
<th>Culture no.</th>
<th>No. of resistant bacteria</th>
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<tbody>
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<td>11</td>
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<td>19</td>
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<td>20</td>
<td>121</td>
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Average: 131.2
Variance: 151.1
Chi-square: 17.3
P: 0.26
some that were only slightly more resistant than the original strain, some that were almost completely resistant, and some that fell between these two extremes. Resistant strains of the second-step, third-step, etc. also showed behavior similar to the first-step strains.

Using the same principle as was used on Page 86 with regard to penicillin, i.e. that mutations in bacteria are caused by genes, this investigator explained the pattern of resistance to streptomycin by postulating that the genes differ greatly from one another in potency. Thus, if a gene of low potency mutates, the first-step resistant strain will have a low degree of resistance, but if the mutation occurs in a highly potent gene, the first-step resistant strain will be highly resistant. Consequently, considerable variation in the degree of resistance is to be expected between first-step strains. For the same reason, a highly resistant strain may be obtained either in one step, by selection of a highly resistant first-step mutant, or in several steps, by selection of mutants of low resistance values.

Alexander and Leidy (1947) also believed that resistant members were present in the bacterial population from the beginning of the infection, so that the emergence of resistance represented a selective process: the survival of a few resistant organisms after the elimination of the sensitive ones. They expressly postulated that resistance, induced after streptomycin treatment, argued against metabolic adap-
tation of the bacteria. To test their hypothesis, they used two experimental methods on various strains of *Hemophilus influenzae*:

(1) Comparison of strains by *in vitro* sensitivity tests, using an inoculum of one million to 1700 million organisms:

After treatment with streptomycin, the strains which thrived in the presence of 1,000 units per ml. *in vitro* became so resistant that 5,000 units per mouse failed to protect them. Therefore, it was concluded that, as a result of a selective process, the very few resistant members, apparently present in all strains, make up most or all of the population in the cultures examined after commencement of treatment.

(2) Comparison of large bacterial populations (142 to 522 billion) for incidence of organisms, in the original cultures, which resist 1,000 units of streptomycin per ml.:

In this phase of the work, large samples of the original strains, prior to therapeutic use of streptomycin, were searched for possible inclusion of resistant forms among them. They found that all the cultures contained a minute fraction of members, which, before exposure to streptomycin, could grow in the presence of 1,000 units per ml. of the antibiotic. In addition, since the resistant state of one strain was transmitted *in vitro* without change in degree through over one hundred subcultures in the absence of streptomycin, these authors concluded that the traits responsible for resistance
of the organisms were apparently inherited. Furthermore, since the bacterial population was relatively small in those patients with mild or moderately severe infections, the possibility of the presence of a significant number of very resistant members was remote; therefore, the emergence of resistance of a strain during treatment of a patient was conditioned, not by a change in the sensitivity of the organisms as a result of streptomycin action, nor by an unusually high initial incidence of organisms with resistant strains, but by the size of the bacterial population.

Yow and Spink (1949) similarly showed the presence of naturally occurring streptomycin-resistant organisms in sensitive strains of *B. abortus* and *Brucella suis* by exposing large bacterial populations to varying concentrations of streptomycin. They found that the number of surviving streptomycin-resistant variants decreased very rapidly as the concentration of streptomycin increased from 1.0 to 5.0 μg. per ml.; then, in response to a concentration of 10,000 μg. per ml., the decline became more gradual after which there were no surviving variants.

Miller and Bohnhoff (1946) observed that resistance to streptomycin developed with such rapidity that two or three transfers onto media containing increasing concentrations of this antibiotic was sufficient to permit meningococci or gonococci to multiply on media containing 50,000 μg. of streptomycin per ml. Meningococci which were rendered strepto-
mycin-resistant by this means retained approximately the virulence of the original parent culture and were resistant to streptomycin in vivo.

Further work by Miller and Bohnhoff (1947b and 1947c) has revealed the presence of two colony types of resistant variants in each of eighteen strains of meningococci, when heavy seedings of meningococci were planted onto a series of plates containing streptomycin in concentrations varying from 40 to 10,000 μg. per ml. One variant, designated types A, appeared in small and approximately equal numbers in all concentrations. It grew in large, yellowish colonies on both streptomycin-free and streptomycin-containing media. In addition, passage of this variant through mice did not cause a decrease in their resistance to streptomycin. The other variant, "B", appeared in greatest numbers on concentrations between 100 and 400 μg. per ml., the concentrations optimal for its multiplication. Although its colonies varied in size and color, depending upon the concentrations of streptomycin on which they developed, the type B variants from any strain were found to be identical. These "B" variants actually required streptomycin for growth both in vitro and in vivo. The characteristics of both types of variants are summarized in Table 22.

In order to explain the origin of these variants, it was assumed that they both arose by current mutation, i.e. from mutants which were constantly appearing in the original
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Type A</th>
<th>Type B</th>
</tr>
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<tbody>
<tr>
<td>Colony size</td>
<td>Large on all concentrations of streptomycin</td>
<td>Small on low concentrations; medium on higher concentrations.</td>
</tr>
<tr>
<td>Colony color</td>
<td>Yellowish</td>
<td>Pearl gray on low concentrations; Slightly yellowish on higher concentrations</td>
</tr>
<tr>
<td>Viability on all concentrations of streptomycin</td>
<td>Grows readily</td>
<td>Grows readily on concentrations above 5 µg/ml.</td>
</tr>
<tr>
<td>Viability on streptomycin-free media</td>
<td>Grows readily</td>
<td>No growth*</td>
</tr>
<tr>
<td>Virulence for mice</td>
<td>Fully virulent</td>
<td>Nonvirulent except in streptomycin-treated mice</td>
</tr>
<tr>
<td>Effect of streptomycin treatment on experimental infection in mice</td>
<td>No protection from maximal doses</td>
<td>Promotes development of fatal meningococcal sepsis</td>
</tr>
</tbody>
</table>

*Among many subcultures onto streptomycin-free media, only four single colonies grew out slowly.
bacterial population of the parent strain. The type A variants developed from any given strain with about equal frequency on all concentrations of streptomycin, with its incidence estimated to average 1 to 3 in $10^{10}$ of the original bacterial population. When the type B variants were first observed, it was thought they were induced by the action of streptomycin; however, subsequent observations have tended to indicate that they, too, originated from mutations which were occurring regularly in the parent bacterial population. Therefore, the rapid development of streptomycin resistance by the meningococci was due to the selective propagation of resistant variants, which became apparent during growth on streptomycin-containing media.

Resistant organisms that require streptomycin for their growth were not confined to the meningococci. Kushnick et al. (1947) made similar observations on strains of *E. coli*, *Pseudomonas aeruginosa*, and *B. subtilis*. They isolated a variant from a streptomycin-resistant strain of *B. subtilis* that required a concentration of 150-300 μg. per ml. of streptomycin, and which grew only anaerobically. The susceptibly parent strain and the streptomycin-requiring variant were tested for their ability to grow in an ammonium chloride synthetic medium to which various sources of carbon were added. Although the susceptible parent strain failed to grow in any of these media containing streptomycin, the streptomycin-requiring variant grew equally well in those
media containing streptomycin with glucose, or glucose and sodium glutamate. Glucose, however, could not replace streptomycin for the growth of this variant.

Paine and Finland (1948) similarly showed that the ability of certain bacteria to survive exposure to an ordinarily lethal concentration was due to the appearance of variants which possessed either of two characteristics: (1) independence of streptomycin activity, i.e. resistance to streptomycin, or (2) dependence upon streptomycin for growth. In this work, streptomycin-resistant and streptomycin-dependent variants were isolated from sensitive strains of S. aureus, E. coli, Proteus morgani, P. aeruginosa, and Klebsiella pneumoniae. Following an initial exposure of an apparently sensitive strain to 1,000 μg. of streptomycin per ml. of agar, some colonies appeared which, on subculture, proved to consist of entirely highly resistant organisms. These organisms remained resistant on subsequent subcultures both in the presence and in the absence of streptomycin. Thus, streptomycin resistance appeared to be a relatively permanent trait and apparently involved the entire progeny of the resistant organism, once it appeared.

Except in the case of P. morgani, the resistant strains showed no difference in their colonial appearance, and, microscopically, showed only slight alteration from the normal. These resistant variants grew equally well in plain broth and in concentrations of streptomycin up to 1,000 μg.
per ml. In addition, a study of various biochemical reactions disclosed no differences between the sensitive bacteria and their derived resistant variants.

Since the resistant variants grew independently of the presence or absence of streptomycin, these authors believed that an alternative mechanism or system existed in the resistant variant which functions in the place of the metabolite or enzyme system that is blocked by streptomycin in the sensitive strain. Furthermore, this may explain the slower growth rate of the resistant variants and why the mutants are not normally encountered until the more rapidly growing sensitive organisms are inhibited by streptomycin.

Klein and Kimmelman (1947) observed that S. aureus, after forty-eight hours' growth in a casein hydrolyzate medium containing streptomycin, showed a marked increase in resistance to this antibiotic. They found that the greater the partially inhibitory action of streptomycin, the greater was the increase in resistance. For example, when bacteria, grown in two units of streptomycin per ml., were subcultured after forty-eight hours, and retested against eight units of the agent, they showed only a relatively small increase in resistance. However, bacteria grown in four and eight units of streptomycin per ml. showed significantly greater increases in resistance.

This role of drug concentration in the development of
streptomycin resistance was explained on the basis of the selection and multiplication of resistant variants. Thus, in high concentrations of streptomycin which did not completely inhibit growth, all but a few of the most resistant bacteria in the initial inoculum would be eliminated. These few bacteria would then multiply and resistant variants would be thrown off in the direction of greater drug resistance. At lower drug concentrations, however, there would not be as effective a selection of the few resistant variants because more of the less resistant bacteria would survive. These latter bacteria, on subculture, would tend to overgrow the few most resistant variants. Therefore, upon re-assaying, such a culture would show only a moderate or slight increase in resistance.

In an attempt to harmonize the metabolic adaptation and mutation theories, Clark and Rantz (1947) made a clear demonstration of the presence of two types of resistance. They showed that colon bacilli from the urinary tract might become resistant in small, orderly steps when they were grown in low, but progressively higher, concentrations of streptomycin. Thus, organisms which grew in 5 µg. but were killed by 10 µg. of the drug per ml. of medium, could, in four steps, be made to grow in 20 µg. per ml. In addition, resistant colonies were isolated on the first transfer of the culture to media which contained drug concentrations
adequate to inhibit the growth of the bulk of the organisms.
Therefore, they drew two conclusions:

(1) Gradual adaptation of the organisms occurs when they are cultured in sub-bactericidal concentrations of streptomycin and then serially transferred to media containing progressively greater amounts of the drug.

(2) On the other hand, very small numbers of excessively resistant variants, or mutants, spontaneously emerge from sensitive strains from time to time.
COMBINED ACTIVITY BETWEEN KEY ANTIBIOTICS AND OTHER CHEMOTHERAPEUTIC AGENTS

Because a single chemotherapeutic agent is not completely effective against an entire bacterial population, the use of several agents of widely divergent chemical structures came into prominence as an approach to the problem. Such a procedure was thought to insure a greater degree of inhibition of metabolic activity in the bacterial cell. Thus, there arose the field of synergistic or additive activity of chemotherapeutic agents.

According to Kolmer (1948) and Price et al. (1949), there appear to be three possible explanations for the effects of combined chemotherapy. In the first place, one compound may aid, cooperate with, or potentiate the other with a material increase of antimicrobial effects, designated as synergistic activity. Thus, the mixture would have a greater effect than the sum of the effects of the active ingredients. Secondly, the enhanced therapeutic activity may be merely additive, i.e. a mathematical summation of the antimicrobial effects of the two compounds acting independently of each other. Thirdly, the mixture may produce an antagonistic effect, i.e. a lesser effect than the sum of the effects of the active constituents.

Sulfa Drugs and Penicillin

Studies by Ungar (1943) showed the enhancing effect of sulphapyridine on penicillin activity. The former drug, when
tested against staphylococcus (strain 663), showed no inhibition of growth in digest broth in dilutions up to 1:2000. However, when sulphapyridine was added to dilutions up to 1:50,000 to a solution of sodium penicillin, which inhibited at 1:30,000, the resulting inhibition took place up to a titre of 1:70,000. When the experiment was repeated using 1% glucose broth, the activity of penicillin against staphylococcus (strain 663) and streptococcus (strain 618) was again shown to be increased. Thus, the inhibitory effect of penicillin in the presence of small quantities of sulphapyridine was at least doubled.

These investigators, having shown in vitro the synergistic effect of penicillin and sulphapyridine, extended their experiments to mice. Thus, groups of six mice were treated with (a) small doses of sulphapyridine insufficient to give any protection against either streptococcus or staphylococcus, (b) small doses of penicillin, and (c) both of these drugs simultaneously. The results, as shown in Table 23, indicate the increased protective effect of both substances given together. These men believed that this enhanced effect was due either to the synergistic action of the two agents on the micro-organisms, or to a chemical reaction between the sulfonamide and the penicillin.

Soo-Hoo and Schnitzer (1944), using beta hemolytic streptococci, confirmed Ungar's observation of the enhanced activity of subtherapeutic doses of penicillin and sulpha-
TABLE 23

EFFECT OF PENICILLIN AND SULPHAPYRIDINE ON STREPTOCOCCUS hemolyticus
AND STAPHYLOCOCCUS aureus IN MICE

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. of mice survivors</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S. hemolyticus</td>
<td></td>
<td></td>
<td></td>
<td>S. aureus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Days</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Sulphapyridine</td>
<td></td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sodium penicillin</td>
<td></td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Sulphapyridine and sodium</td>
<td></td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>penicillin</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>Control</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Treatment stopped

161
pyridine in vivo. They proposed that the potentiation or synergism between these two different agents resulted because of a different mechanism of activity of penicillin on one side, and of sulphapyridine on the other.

Bigger (1944) showed the superiority of sulphathiazole over sulphanilamide or sulphapyridine in greatly increasing the dilution at which the inhibitory action of penicillin on Staphylococcus pyogenes and streptococcus pyogenes could be demonstrated. He suggested that the value of sulphathiazole, when employed to reinforce penicillin, appeared to lie in its power to render effective a concentration of penicillin which alone was either ineffective or only partially effective against certain micro-organisms. Furthermore, this author noticed that the degree of synergic action varied considerably with the strain of the organism, but was obvious even when no inhibitory action of sulphathiazole alone could be detected.

Further evidence to support the belief that the action of penicillin was considerably enhanced by certain sulphonamides was given by T'ung (1944). He found that the combination of penicillin with small amounts of sulfathiazole resulted in a considerably greater antibacterial action on organisms of the Brucella group than penicillin alone. Like Bigger, T'ung also found this synergistic action to be more noticeable on certain strains than on others.

Thomas and Hayes (1947), in studying the synergic action
of penicillin and sulpha-thiazole on S. typhosa, observed that the presence of sulpha-thiazole caused the number of organisms in the inoculum to fall to a very low level. Furthermore, the lethal action of penicillin increased as the inoculum decreased. Thus, they postulated that the reduction in the number of organisms by sulpha-thiazole brought them within the effective range of penicillin, which alone was unable to cope with the original inoculum.

Massell et al. (1946) studied the combined action of a sulfonamide (sulfa-thiazole, sulfadiazine, and sulfamerazine) and penicillin on the viridans streptococci. Sulfonamide drugs had the following effects on the in vitro action of penicillin: (a) the minimal effective concentration of penicillin necessary to produce both bacteriostasis and destruction of the organisms was reduced in many strains. For example, a concentration of 0.11 units of penicillin, which had been ineffective in the absence of sulfamerazine, in its presence caused almost as much destruction of bacteria as 0.23 units. (b) However, in the presence of penicillin in amounts above the minimal effective concentration, sulfonamides did not appreciably alter the bactericidal action of the penicillin. Thus, an increase in the concentration of penicillin from 0.23 units to 0.91 units per ml. did not accelerate the rate of destruction of the bacteria in the presence of sulfamerazine. These experiments emphasized the important role played by the concentration of the chemo-
therapeutic agents in synergistic or additive activity.

Kirby (1944b) found that sulfadiazine and penicillin, in concentrations which were not bacteriostatic separately, did not cause inhibition of growth of staphylococci when mixed together. However, if concentrations were used that were bacteriostatic separately, sulfadiazine-penicillin mixtures produced greater bacteriostasis than did any of these substances alone. Furthermore, in no instance was the bacteriostatic action of the mixture greater than the sum of the inhibitory effects of the individual constituents. In other words, there was no actual synergism or potentiation; the effects were merely additive.

Similarly, Klein and Kalter (1946) found that the in vitro combination of sulfathiazole, sulfadiazine, or sulfapyridine and penicillin resulted in an additive effect in which both agents appeared to inhibit separately. In their experiments, they used five sulfonamide-susceptible and five sulfonamide-resistant strains of S. aureus, and eight gram-negative rods. Although the sulfonamide played a comparatively quiescent role in the first 10-12 hours of growth, it became actively bacteriostatic between 12 and 48 hours. This combined action of penicillin and a sulfonamide was explained as resulting from an initial reduction in the total number of bacteria by the lytic action of penicillin. This permitted a concentration of sulfonamide, which was only partially inhibitory in the presence of a large number of
cells, to become completely inhibitory in the presence of a smaller number of cells.

According to Hobby and Dawson (1946), the combined use of penicillin and sulfadiazine was dependent on many factors. Among these were (1) the concentration of each bacteriostatic agent, (2) the number of organisms present, (3) the environmental conditions allowing growth of the organisms, (4) the degree of susceptibility of the organisms to penicillin and to sulfadiazine, and (5) the individual species of organisms involved.

These men found that hemolytic streptococci, which were inhibited only temporarily by penicillin and were not inhibited by sulfadiazine, could be inhibited permanently by combined drug action under certain experimental conditions. For example, sulfadiazine, in the presence of little or no bacteriostatic amounts of penicillin, increased bacteriostasis provided that the organism was sulfadiazine-sensitive and that it was present in small numbers only. On the other hand, in the presence of larger amounts of penicillin, the bactericidal action was very rapid during the first few hours of incubation. However, if sterilization was not complete at the end of 5-7 hours, the presence of sulfadiazine, under the same experimental conditions as above, prolonged the lag period, thus decreasing the multiplication rate of the streptococci. This, in turn, decreased the bactericidal rate at which penicillin acted.
This latter observation of a sulfonamide-penicillin antagonism had been previously demonstrated by Garrod (1944). He found that, in vitro, the presence of a bacteriostatic concentration of sulphathiazole reduced the velocity of penicillin action on staphylococci by about one-half.

Furthermore, Cohn and Seijo (1944) reported that the growth of sulfonamide-resistant strains of gonococci were unaffected by combinations of subtherapeutic doses of penicillin and sulfathiazole. In order to show this, they carried out two sets of experiments: in the first, 11 sulfonamide-resistant strains were grown in bloods containing 3 mg. per 100 ml. of sulfathiazole and penicillin in a dilution of 1:200,000 or 1:500,000. In the second experiment, three resistant strains were first grown in these same dilutions of penicillin for twenty-four hours and then transferred to blood containing 3 mg. per 100 ml. sulfathiazole. The results showed that the growth of the gonococci strains was not affected in either of the two experiments.

**Sulfa Drugs and Streptomycin**

Smith et al. (1947) studied the combined effect of streptomycin and sulfones on tuberculosis that was produced experimentally. They found that the efficacy of the combination was greater in every instance than the sum of the effects of the individual components. Since large amounts of evidence had been previously accumulated to indicate that
bacteria can acquire a high degree of resistance to streptomycin, these investigators postulated that the mechanism to explain the synergistic action of the two agents consisted of the elimination or attenuation of strains which might acquire a resistance to streptomycin.

Klein and Kimmelman (1947) also showed that the greater effectiveness of sulfadiazine as a synergist was related to its ability to reduce more effectively the resistance of bacteria surviving the action of streptomycin. Thus, bacteria grown in streptomycin alone showed a very marked increase in resistance, whereas bacteria grown in a combination of streptomycin and sulfadiazine showed only a moderate increase in streptomycin resistance.

Further work by Shaffer and Spink (1948) was presented which showed that a synergism resulted from the combined action of sulfadiazine and streptomycin in experimental Brucella infection of the developing chick embryo. When streptomycin was used alone, it gave incomplete protection and eliminated the infection from only 13% of the embryos; sodium sulfadiazine also only gave partial protection when used alone. However, combined therapy eliminated the infection from 87% of the embryos. These men believed that the primary mechanism or significance of the streptomycin-sulfadiazine synergism was not only control of the infection, but the elimination of Brucella from the tissues of the host.
Streptomycin and Penicillin

Kolmer (1948) showed that mixtures of penicillin and streptomycin were capable of synergistic activity of S. aureus. For example, the smallest amount of penicillin giving complete inhibition of the H strain of S. aureus was 0.2 unit, while the minimal inhibitory concentration of streptomycin was 0.001 mg. (1 unit). Mixtures of the two compounds indicated synergistic action since the minimal inhibitory concentration was 0.05 unit of penicillin and 0.0005 mg. (0.5 unit) of streptomycin. Since streptomycin inhibited the metabolism of resting cells, whereas penicillin acted chiefly on the actively proliferating organisms, it was possible that, with both drugs, bacteria in all phases might be affected.

Jawetz et al. (1950) reported a synergistic effect of penicillin-streptomycin mixtures in vitro against certain enterococci. Streptomycin, in concentrations of 25 to 50 µg. per ml., completely failed to inhibit a large bacterial inoculum, while the concentration of penicillin that was used was optimal for the particular organism. When streptomycin was added to penicillin, two striking effects were noted: (1) the rate of bactericidal action was greater than with penicillin alone, and (2) the mixture of the two drugs usually produced complete sterilization of the medium, so that within three to five days all enterococci were non-viable.
and failed to grow in subculture even after adequate amounts of penicillinase had been added. Thus, these authors concluded that the main demonstrable effect of streptomycin-penicillin synergism on enterococci was an increase in the rate of bactericidal action beyond the optimum obtainable with penicillin alone. Furthermore, since organisms to which penicillinase had been added were found to be equally susceptible to the action of streptomycin as the rest of the population, they rejected the hypothesis that streptomycin-penicillin mixtures might be explained on the basis of inhibition of penicillin-resistant strains by streptomycin.

**Streptomycin, Penicillin, and Sulfadiazine**

Klein and Kimmelman (1947) studied the combined action of 4 units of streptomycin, 0.04 unit of penicillin, and a 1:10,000 dilution of sulfadiazine against S. aureus. Although this combination of drugs completely inhibited growth, there were always a few surviving bacteria. These latter bacteria, when subcultured and reassayed against streptomycin, penicillin, and sulfadiazine, respectively, never showed any increase in resistance to these agents, and regularly showed a slight decrease in their growth rate in the presence of streptomycin and occasionally a slight decrease in resistance to penicillin and sulfadiazine. The absence of any increase in resistance to this mixture of drugs was interpreted as resulting from the prompt inhibition of all multiplication with the sub-
sequent inability of resistant variants to arise. Thus, they believed that the few surviving bacteria represented non-dividing or resting cells in a physiological state temporarily unaffected by the action of the drugs.

Penicillin and Bacitracin

Eagle and Fleischman (1948) described the synergistic action of penicillin and bacitracin (an antibiotic derived from culture filtrates of the Tracy strain of *B. subtilis*) against *T. pallidum* in rabbits. Whereas 9,000 units/kg. of bacitracin were necessary to cure 50% of the animals (CD$_{50}$), a similar curative dose of penicillin was about 30-40 mg./kg. However, when as little as 1 mg./kg. of penicillin was administered simultaneously with bacitracin, the curative dose of bacitracin was reduced from 9,000 to 1,280 units/kg. Thus, the two drugs were as much as seven times more effective when they were used in combination. A further increase in the dosage of penicillin from 1 to 4 to 16 mg./kg. caused a further progressive decrease in the CD$_{50}$ dose of bacitracin from 1,280 to 840 to 480 units/kg.

Bachman (1949) studied the in vitro effect of penicillin and bacitracin mixtures against several strains of $\alpha$- and $\gamma$-hemolytic streptococci. He found that when these agents were used in combination, growth inhibition could be affected with a very low concentration of each. However, neither penicillin nor bacitracin alone, even when their concentrations were
doubled, could prevent the growth of the organisms. Thus, this author proposed that organisms susceptible to penicillin and/or bacitracin may be expected to respond much more rapidly to a mixture of these antibiotics with a resulting synergistic effect.

This same investigator believed that it was desirable to use combinations of antibacterial substances in order to combat the development of bacterial resistance. He theorized that, since resistance to antibiotic A was probably inherited independently of antibiotic B, the chances were very small that one or two cells per million, which were resistant to antibiotic A, were also resistant to antibiotic B. Therefore, when antibiotics A and B were used together, each in a concentration which inhibited the multiplication of all but the resistant organisms, there would be no survivors, except in tremendous populations.

**Penicillin and Gramicidin**

Heilman and Herrell (1942), using a tissue culture technique, studied the effect of a mixture of penicillin and gramicidin (one of the two active principles of the antibiotic thyrothricin) on *Diplococcus pneumoniae* type VII. Penicillin and gramicidin were not mixed before use, but were added separately to the tissue culture medium. These investigators observed it was necessary to use almost a full effective amount of each substance in order to cause total inhibition
when the two drugs were used together. Thus, they concluded that the antibiotics complemented each other only to a very slight extent, so that their antibacterial effect was only slightly more than additive.

Foley and Lee (1948) studied the effect of mixtures of gramicidin and penicillin on different organisms. When *S. aureus*, relatively resistant to gramicidin, was used, 0.7 µg. of penicillin and 5.0 µg. of gramicidin per ml. killed staphylococci at a faster rate than did penicillin alone at 0.7 µg./ml.

The coaction of penicillin and gramicidin was then studied using a hemolytic streptococcus of Lancefield's Group D. This organism was chosen because it was relatively resistant to penicillin, but could be inhibited by small amounts of gramicidin. Penicillin alone (35 µg. per ml.) and gramicidin (0.5 µg. per ml.) were not bactericidal. However, when these two antibiotics were mixed together, in these same concentrations, they were bactericidal.

Finally, it was important to study the coaction of penicillin and gramicidin on an organism which was highly susceptible to both these agents. Therefore, a strain of *Streptococcus hemolyticus* (Group B) was chosen. Whereas 0.5 µg. of gramicidin per ml. killed at a faster rate than 0.70 µg. of penicillin, an even more marked bactericidal action was noted under the influence of a mixture of the two drugs.
The results of these experiments, in which cocci were exposed to mixtures of penicillin and gramicidin, suggested to these men that the antibiotics attacked separate systems of the organisms. This was shown by the differences in the rate of killing by the mixtures over that obtained when the agents acted separately, and by the differences in susceptibility of the various organisms that were tested.

Penicillin and Chloromycetin, Aureomycin, or Terramycin

Jewetz et al. (1950), while studying the effect of a mixture of chloromycetin (10 µg./ml.) and penicillin (6 µg./ml.) in vitro on the enterococci, found that the bactericidal rate of the mixture was less than when penicillin was used alone. Thus, it appeared that chloromycetin somehow interfered with the early bactericidal effect of penicillin, i.e., had some action antagonistic to penicillin.

Jewetz and Speck (1950), in a continuation of this work, were interested in determining whether this apparent antagonism between penicillin and chloromycetin could be demonstrated in vivo. Therefore, groups of twenty mice were infected with streptococcus C 203, and were treated with either penicillin alone (30 µg./ml.), chloromycetin alone (800 µg./ml.), or a mixture of both drugs. Whereas either drug alone protected all but 20% of the animals, the administration of both drugs together resulted in a mortality of 60%. It was, therefore, apparent that these two antibiotics
interfered with each other's efficacy as therapeutic agents. When chloromycetin and penicillin, either alone or in combination, were administered to uninfected mice, there was no evidence of toxicity. Consequently, the higher death rate encountered with the joint use of both drugs could not be attributed to toxic depression of the host's defense mechanism, nor to direct toxic action of the mixture.

The quantitative relationships of this joint action of drugs was then investigated by keeping the concentration of one agent constant and varying the other. The results showed that increasing doses of chloromycetin with a constant dose of penicillin greatly increased the death rate of the mice. However, when the dose of chloromycetin was kept constant and the dose of penicillin varied, the death rate was lower. Therefore, these authors concluded that chloromycetin in some way quantitatively interfered with the effect of penicillin.

Three hypotheses were presented to explain how this drug interference or antagonism might take place. (1) The two drugs might interact with one another in some physical or chemical process, so that the resulting complex would be of lesser chemotherapeutic efficacy. (2) The two drugs might compete with one another for "receptors" on the susceptible bacterial cells, so that, by means of mass action, the less effective drug would interfere with the more potent one. (3) Possibly one drug might so change the developmental
characteristics or properties of the infected micro-organism that their susceptibility to other agents would be greatly diminished.

Gunnison et al. (1950) studied the interference of aureomycin and terramycin with the action of penicillin on S. pyogenes and K. pneumoniae. When S. pyogenes was used, 0.006 μg. of penicillin per ml. was rapidly bactericidal. On the other hand, aureomycin or terramycin, in concentrations of 1-10 μg. per ml. were mainly bacteriostatic. When either of these less active drugs was added to penicillin, the death rate of the streptococci approximated that obtained with the less effective antibiotic alone. An increase in the concentration of aureomycin or terramycin to 10-50 μg./ml. resulted in a slight increase in their antagonism towards penicillin. However, when the concentration of one of these "newer" antibiotics was further increased to a point where it was moderately bactericidal by itself, interference with penicillin could no longer be demonstrated. Therefore, apparently aureomycin and terramycin interfered with the action of penicillin only in those concentrations which were at least bacteriostatic and yet not markedly bactericidal by themselves. Consequently, they proposed that inactive concentrations and highly active concentrations of aureomycin and terramycin did not antagonize the activity of penicillin.

In the case of K. pneumoniae, similar concentrations of
the three antibiotics were moderately active in about equal
degree. For example, the death rate of this organism in the
presence of 10 µg. of aureomycin per ml. was the same whether
or not a bacteriostatic concentration of penicillin was added.
Therefore, since no interference was observed, it seemed un-
likely that the antagonism of one antibiotic by another was
due to a chemical inactivation without reference to the
sensitivity of the bacteria concerned.

A possible mechanism to explain this antagonistic action
between antibiotics was drawn from the idea that, while
penicillin principally affected dividing organisms, aureomycin,
chloromycetin, and terramycin may depress bacterial multi-
plication. Such an interference with multiplication would
result in interference with penicillin action.

The fact that all three of the "newer" antibiotics that
were widely used clinically may interfere with the bactericidal
action of penicillin was of interest to these writers for two
reasons. First, this demonstration of antagonistic relation-
ships between antibiotics would draw attention to the in-
advisability of the indiscriminate, concurrent use of several
antibiotics. Furthermore, the observed incompatibility between
drugs might provide a useful laboratory tool for the in-
vestigation of antibiotic activity, provided that it can be
shown that the antagonism was not due to a simple chemical
inactivation, but to an interference between the drugs at
their site of action.

In conclusion, it is interesting to note the studies of Price et al. (1949) on the combined action of antibiotics and other agents. They found that, although a synergistic action was demonstrated with a number of combinations of chemotherapeutic agents, in only 50% of these combinations did the in vitro methods correlate with the in vivo tests. This emphasized the importance of animal and human studies in spite of the limitations and cautions required in interpreting the results observed in the treatment of the infectious diseases of man.
SUMMARY

1. Penicillin, a ring condensation of two amino acids, has various effects on bacteria.

2. Micro-organisms that are exposed to varying concentrations of penicillin show striking morphological and cytological changes, such as elongated and swollen cells, dissimilation of the cell wall, "diplococcus-like" structures, and bizarre involution forms.

3. Most of the evidence indicates the importance of the sulfhydryl group in bacterial metabolism.

4. Exposure to bacteriostatic concentrations of penicillin may be interpreted in terms of a shift of sulfhydryl (-SH) to disulfide (S-S), aldehyde CHO to carboxylic COOH or ketonic CO, or enolic COH to ketonic CO.

5. Penicillin may compete with glutathione for enzymatic or other mechanisms important for microbial reproduction.

6. Penicillin interferes with the action of ribonuclease and phosphatase and thus affects nucleic acid metabolism.

7. The ability of various micro-organisms to ferment disaccharides and to bring about the dephosphorylation of adenosine triphosphate is inhibited by penicillin; furthermore, lipids are displaced from bacterial cells and hydrolyzed into fatty acids.

8. Penicillin, under certain conditions, may stimulate the growth of cells in the vicinity of the dying micro-
organisms.

9. Most authors believe that penicillin acts chiefly on dividing cells.

10. Although publications relating to the effect of pH on penicillin activity are contradictory, it appears that the activity of penicillin increases as a result of a lowering of the pH; this effect may result from a competition between the acid anions of penicillin and the hydroxyl ions of the culture medium.

11. In some bacteria, penicillin resistance may be definitely ascribed to their ability to produce penicillinase, a penicillin-destroying enzyme.

12. Others believe that resistance to penicillin is an inherited characteristic which originates through mutation, and whose origin is independent of contact with the antibiotic.

13. Streptomycin, a basic glycoside, has three potential active groups: (a) guanidine groups, (b) free amino groups, and (c) a carbonyl group.

14. Streptomycin, like penicillin, causes definite alterations in the size and shape of various bacteria.

15. The latest evidence indicates that streptomycin prevents the formation of an hypothetical intermediate necessary for the amino acid-metabolizing system of *E. coli*; this, in turn, prevents the "terminal respiration" process in this organism and inhibits the oxalacetate-pyruvate
condensation.

16. Streptomycin inhibits the oxidation of certain carbohydrates and carbohydrate intermediates, and of the breakdown products of higher fatty acids.

17. The inhibition of benzoic acid oxidation by streptomycin may be primarily due to interference with the formation of benzoic acid oxidase.

18. Streptomycin, at certain concentrations, may stimulate the growth of certain pathogens.

19. An increase in the pH of a solution markedly increases the activity of streptomycin; this may be due to competition between the base cations of streptomycin and the hydrogen ions of the culture medium.

20. Although there is very little evidence for the production of an enzyme comparable to penicillinase, resistance to streptomycin may be developed as a result of metabolic adaptation by the bacteria and/or through mutation.

21. Synergistic or additive effects are shown by the following combinations of drugs: (a) Sulfadiazine and penicillin or streptomycin; (b) penicillin and streptomycin, bacitracin or gramicidin.

22. The "newer" antibiotics, chloromycetin, aureomycin, and terramycin, appear to be incompatible with the bactericidal action of penicillin.
OUTLOOK FOR THE FUTURE

Sulfonamides were in general use for about five years before the nature of their action on bacteria was discovered. The history of penicillin and streptomycin has developed along similar lines. Since in vitro and in vivo experiments have demonstrated the remarkable properties of these antibiotics, and thus encouraged clinical trial, the treatment of human disease by these agents was embarked on at an early stage and has engrossed almost all attention. However, the far-reaching success of these efforts did not diminish the need for a clearer understanding of the mode of action of these antibiotics on micro-organisms. Such knowledge has been forthcoming, but still further information must be gathered in order to enable clinical treatment to be directed even more effectively. For example, in my review of the literature, I have not been able to find any experimental evidence to indicate which group or groups of the penicillin molecule were active in bacterial inhibition.

In the past few years, there has been an increasing desire on the part of research investigators to discover if new chemotherapeutic agents could be developed which would attack not only the common bacterial invaders, but also the pathogens in the viral, rickettsial, and protozoan groups. Complete success in this direction has not yet been achieved, but may be said to be gradually coming into view. An inter-
mediate step has been accomplished with the discovery of three of the "newer" antibiotics, all derived from the genus Actinomyces: chloromycetin (Ehrlich et al., 1947), aureomycin (Duggar, 1948), and terramycin (Finlay et al., 1950). History has again repeated itself for, despite the widespread clinical research which has been done on these antibiotics and on other new chemotherapeutic agents, few publications have appeared on their modes of action.

However, it is safe to assume that, during the next few years, the "modus operandi" of these agents will be revealed by biochemists, geneticists, microbiologists, pharmacologists, and physiologists from experimental investigations now in progress. Thus, it may definitely be concluded that the field of antibiotics, in spite of the tremendous progress made during the past decade, may still be considered in its embryonic stage awaiting further development and organization.
ABSTRACT

An antibiotic is an organic substance produced by a living organism which inhibits the growth of or kills some other living organism. Two of the more important antibiotics that have been studied are penicillin and streptomycin.

Although three structural formulae for penicillin have been presented, this antibiotic is generally thought of as a ring condensation of two amino acids: alanine and beta-dimethylcysteine. The various penicillins differ in the substituent acid group coupled to the alanine amino group.

Most investigators agree that penicillin produces striking and bizarre changes in the shape and size of various bacteria. The presence of elongated and swollen cells suggests the idea that growth takes place, but fission fails to follow. Other cultures contain cells which show a tendency to change from the normal three-dimensional colonies to the formation of "streptococcus-like" and "diplococcus-like" structures.

Penicillin has been shown to produce various alterations in bacterial metabolism. Most of the evidence agrees with the theory that penicillin either blocks the normal functioning of protein -SH groups so that the -SH ⇌ S-S equilibrium is not normal, or exposes these sulfhydryl groups so that they react with penicillin. As a result, there is no longer available to the aerobic cell a reservoir of hydrogen (controlled by dehydrogenases) which would rehydrogenate diketones to dienols. Therefore, it is possible that essential poly-
peptides of the glutathione type (containing an \(-\text{SH}\) group) may compete with penicillin for enzymatic or other mechanisms important for microbial reproduction. In addition, penicillin inhibits enzymes, such as ribonuclease and phosphatase, which are important in nucleic acid metabolism.

Very little work has been done to determine the effect of penicillin on carbohydrate and fat metabolism. However, it has been shown that penicillin interfered with the fermentation of disaccharides, such as galactose; furthermore, lipids were displaced from cells undergoing lysis and were hydrolyzed into fatty acids.

Two theories have been proposed to account for the narrow ring of enhanced growth that was observed in penicillin assay plates. The increase in metabolism resulted either from direct stimulation of cells in this region or from "growth factors" which diffused from lysed cells in the inhibition zones.

Most authors agree that the action of penicillin is chiefly on dividing cells. Furthermore, as a result of the bactericidal action of penicillin, the organisms become more vulnerable to autolysis.

Publications relating to the effect of pH on penicillin activity are contradictory. By different methods, penicillin was shown to increase its activity as a result of lowering or raising the pH. The theory that penicillin activity is
due to a competition between the acid anions of penicillin and the hydroxyl ions of the culture medium favors the hypothesis that penicillin inactivation results from an increase in pH.

Two theories have been proposed to explain the origin of bacterial resistance to penicillin. Although the enzyme, penicillinase, may, in certain instances, play an important role in the development of resistance, there are too many inconsistencies in the literature to accept this theory as the sole means of explaining penicillin-resistance. Therefore, experimental evidence was presented which favored the theory that resistance originated by mutation with penicillin acting only as a selective agent which destroyed the sensitive bacteria and thus isolated the mutants.

The structure of streptomycin indicates that it is a basic glycoside, which consists of two main portions: (1) streptidine and (2) streptobiosamine. Three groups of the streptomycin molecule appear to be essential for streptomycin activity: (a) guanidine groups, (b) free amino groups, and (c) a carbonyl group. Evidence was presented on both sides to determine the essentiality of each one of these groups in streptomycin activity.

Little attention has been directed towards possible changes in bacterial morphology as a result of growth in the presence of streptomycin. It was suggested that the observed
structural changes, such as increase in length and diameter of the cells, may be secondary effects resulting from disturbances of intermediary metabolism.

The latest work on the effect of streptomycin on bacterial metabolism is centered around the fact that streptomycin prevents the "terminal respiratory" process in susceptible strains of E. coli, thus inhibiting the oxalacetate-pyruvate condensation. Different types of systems were studied in which this condensation would be evident in different manners: (1) the oxidation of keto acids derived from amino acids, (2) the oxidation of fumarate and pyruvate, and (3) the comparison of oxalacetate and pyruvate oxidation by fresh cell and older cell suspensions.

Unlike penicillin, streptomycin inhibits the oxidation of carbohydrate and carbohydrate intermediates by both resting and multiplying bacteria. Results of the effect of streptomycin on fat metabolism indicate that only the oxidation of the breakdown products of the higher fatty acids was affected. Other experimental data has indicated that streptomycin inhibits the formation of the benzoic acid oxidase that is necessary for the oxidation of benzoic acid. However, at certain concentrations of streptomycin, it is possible to obtain an increase in the endogenous respiration of different organisms.

It is fairly well established that raising or lowering the pH of the medium increases or decreases, respectively,
the activity of streptomycin. This reaction effect may be due to competition between the hydrogen ions of the culture medium and the base cations of streptomycin for centers on the cell surface.

As in the case of penicillin, there are two theories to explain the origin of bacterial resistance to streptomycin. Evidence is given to show that, as a result of contact with streptomycin, bacteria become adapted metabolically to the presence of the antibiotic. However, many investigators support the theory that resistant strains arise through mutation, independent of the action of streptomycin. Since an attempt has been made to harmonize both theories, it is possible that resistance to streptomycin is a result of both metabolic adaptation and mutation.

Synergistic, additive, or antagonistic effects may result from treatment with different combinations of chemotherapeutic agents. Investigations within the last few years have been concerned with the synergistic or additive effects of (1) sulfa drugs and penicillin or streptomycin, and (2) penicillin and streptomycin, bacitracin, or gramicidin. Among the various factors which controlled the results were (1) the concentration of each agent, (2) the number of organisms present, (3) the environmental conditions allowing growth of the organisms, (4) the degree of susceptibility of the organism to each drug, and (5) the individual species
that was involved.

Present experiments on mixtures of penicillin and chloromycetin, aureomycin or terramycin indicate that the "newer" antibiotics may be incompatible with the bactericidal action of penicillin. A possible mechanism to explain this antagonistic action was drawn from the idea that, while penicillin principally affected dividing organisms, aureomycin, chloromycetin, and terramycin may depress bacterial multiplication. Such an interference would result in interference with penicillin action. It is also possible that the two drugs might compete with one another for "receptors" on the susceptible bacterial cells, so that, by means of mass action, the less effective drug would interfere with the more potent one.
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