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# Tennecetin: A New Antifungal Antibiotic

James Burns
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To the Graduate Council:

I am submitting herewith a thesis written by James Burns entitled "Tennecetin: A New Antifungal Antibiotic." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

Frank Holtman, Major Professor

We have read this thesis and recommend its acceptance:

Samuel Tipton, Orvin Mundt, Howard E. Reed, & Raymond W. Beck

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

August 1, 1959

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Frank Holtman
Major Professor

We have read this thesis and recommend its acceptance:

Samuel R. Tipton

J. Orvia Mandt

Howard E. Reed

Raymond W. Beck

Accepted for the Council:

Dale Kaufling
Dean of the Graduate School

# TENNECETIN: A NEW ANTIFUNGAL ANTIBIOTIC

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A THESIS

Submitted to
The Graduate Council
of
The University of Tennessee
in
Partial Fulfillment of the Requirements
for the degree of
Doctor of Philosophy

---

by

James Burns

August, 1959

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## DEDICATION

Your work too is here in every line.
(You finished your part before I finished mine.)
Now in lieu of that long-awaited graduation kiss,
Dear Lucia, let me give you this.

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# CHAPTER I

## INTRODUCTION

> A remark concerning Columbus applies to those of us who are in antibiotic research. It was said of him that when he left Spain he did not know where he was going. When he landed, he did not know where he was. When he returned, he did not know where he had been.
>
> Ethan Allan Brown (1955)

The present investigation is an outgrowth of some speculations we have entertained (and vice versa) for a number of years. These speculations revolved around the general idea of antibiotics -- their discovery, production, uses, and significance.

Despite a keen fascination with the matter, our attempts to build a philosophy of antibiosis suffered from an awkward ignorance of the subject itself. There seemed to be no easy way to remedy this situation. In a field dominated by commercial interests, with a literature too recent and scattered to have been sifted and sorted, the traditional academic tools of learning did not appear, in themselves, to be entirely adequate. What was wanted, it seemed, was a maiden voyage on uncharted seas. If such a voyage did not turn out to be one of discovery, it would at least be one of exploration. In less romantic terms, this "voyage" must be an actual experience with a single

antibiotic, preferably one not previously studied, and the experience must extend from the very beginning -- i.e., the discovery of the antibiotic itself -- to whatever fulfillment of understanding might be possible within the limits of our abilities and resources.

Accordingly, some organisms were isolated from nature and studied for their ability to produce an antibiotic substance. Happily, one of the first organisms so studied appeared unusual and interesting from two standpoints: It appeared to be a species of Streptomyces with which we were entirely unfamiliar, and it produced an antibiotic active against yeasts and molds. When we became convinced that we were dealing with an antibiotic not previously described, and with an organism which could not be identified as a previously listed species, we gave the name "tennecetin" to the former and Streptomyces chattanoogensis to the latter.

What is offered here, then, is an account of this first adventure into the field of antibiotic discovery and development. If at times it appears the author is long on adventure and short on scholarship, we submit that it could not have been the other way around.

# CHAPTER II

## LITERATURE SURVEY

> Incidently, one hears lately of applicants for academic positions who ask what the working hours are.
>
> Austen M. Brues (1955)

Although numerous examples of antibiosis may be found in the literature prior to 1928, it is customary and proper to consider Fleming's discovery of penicillin (Fleming, 1929) as the first indisputable landmark of the antibiotic age. The remarkable story of the development of penicillin is so well known as to need no repeating here. It is well to remember, however, that a period of approximately fifteen years elapsed between Fleming's original discovery and the first large scale use of penicillin as an effective therapeutic agent. During this period, this embryonic time of the antibiotic age, much research activity on antibiotics took place.

Penicillin and most of the well known antibiotics that have followed it are principally antibacterial agents. It is interesting to note that antifungal antibiotics, of which there are fewer and less illustrious examples, have essentially the same antecedents in time. For in the same year that Fleming discovered penicillin, Chambers and Weidman (1928) reported on a strain of Bacillus subtilis

antagonistic to certain dermatophytes. The first research group to work on penicillin after Fleming was that of Raistrick, who studied methods for large scale production and the chemical nature of penicillin (Clutterbuck et al., 1932). In that same year Weindling (1932) discovered the antifungal properties of a mold, which he later showed to be due to a specific antibiotic substance, gliotoxin (Weindling, 1934).

The discovery of tyrothricin, an antibacterial agent produced by a soil bacillus (Dubos, 1939) gave impetus to the search for antibiotics; also in 1939 Chain and Florey and their co-workers in England were re-examining penicillin, and the Rutgers University group under Selman A. Waksman began a systematic search among the actinomycetes for antibiotics. At the same time Raistrick's group reported on the structure of griseofulvin, an antifungal agent produced by certain species of Penicillium (Oxford et al., 1939) and Cordon and Haenselar (1939) discovered a bacterium antagonistic to the fungus Rhizoctonia solani. One of the first substances studied by Waksman's group was actinomycin, an antibiotic which possessed some antifungal as well as antibacterial properties (Waksman and Woodruff, 1940).

Despite the promise of these early investigations, however, the development of useful antifungal antibiotics

has not kept pace with the development of antibacterial agents. This is perhaps a reflection of a greater preoccupation in general with bacterial infections than with mycotic infections in man. This situation has already been well stated:

> The discovery of the causal relation of certain of the fungi to infectious disease preceded the pioneer work of Pasteur and Koch with the pathogenic bacteria by several years, for Schoenlein studied the fungus causing favus in 1839, and in the same year Lagenbeck described the yeast-like microorganism of thrush. In spite of its earlier beginnings, medical mycology was soon overshadowed by bacteriology and has never received as much attention through some of the fungus diseases are among the more common infections of man. (Jordan and Burrows, 1945).

But the medical bias toward the bacteria has not been shared by the plant pathologist and other agriculturists and technologists who have long studied the fungi which infect and infest man's crops, his stored grains and foods, his leather goods, and his domestic animals as well as man himself. It is not surprising then, that onee the efficacy of antibiotics in the control of human diseases was established, these workers actively sought antibiotics effective against fungi.

There was already ample evidence that a great many microorganisms isolated from soil have the ability to produce antifungal substances <u>in vitro</u>. Thus Alexopoulos (1941) found that forty-five of his eighty actinomycete

isolates inhibited the growth of Colletotrichum gloeosporoides. These studies were extended to demonstrate inhibition of nine other species of fungi by these same actinomycetes (Alexopoulos and Herrick, 1954).

Similar surveys have been made from time to time. Burkholder (1946) reported that 7 per cent of 7,369 strains of actinomycetes tested for antibacterial activity were also active against Candida albicans. Schatz and Hazen (1948) found 51 per cent of their isolates to be active against one or more test fungi. Aiso et al. (1952a) tested about 400 strains of actinomycetes for antifungal activity; 61 per cent of their isolates were active against at least one of seven test fungi. Landerkin et al. (1950) tested 660 actinomycetes isolated from soils in Northern Canada for antifungal activity; 17 per cent inhibited Helminthosporum sativum, 12 per cent inhibited Fusarium culmorum, 9 per cent inhibited Fusarium lini, and 6 per cent inhibited all three test organisms. Similar results were obtained by Cooper and Chilton (1947; idem, 1949) with organisms isolated from soils in Louisiana against members of the genera Pythium and Rhizoctonia.

In still another screening program, it was found that 315 of 764 actinomycetes and 178 of 315 molds produced substances inhibiting at least one of six pathogenic fungi (Emerson et al., 1946). An interesting sidelight

of this investigation was the finding that all but one of the fifty-eight actinomycetes studied for their antifungal properties inhibited the growth of the human pathogen Cryptococcus neoformans -- an organism which remains to this day refractory to all antibiotics used clinically.

The first useful antifungal antibiotic to come from these screening programs was cycloheximide, originally described under the name actidione (Wiffen et al., 1946; Ford and Leach, 1946). This antibiotic, produced by Streptomyces griseus, has been the subject of much study (Wiffen, 1947; idem, 1948; idem, 1950; Kornfield et al., 1949). Its use in human infections is limited by its toxicity and by the peculiar circumstance that whereas it has good activity against most saprophytic fungi, it is relatively inactive against most of the fungi capable of causing disease in man and animals. This characteristic has been made use of in the development of a culture medium for the selective isolation of pathogenic fungi from clinical sources (Georg et al., 1954). Cycloheximide has found limited use in the control of fungus infections in plants.

Other early antifungal antibiotics which were developed with agricultural purposes in mind are antimycin A (Leben and Keitt, 1948; Dunshee et al., 1949) and endomycin (Gottlieb et al., 1951). Antimycin A has had

some use as an antifungal agent against plant diseases (Leben and Keitt, 1954), and has been found to be an inhibitor of certain respiratory enzymes (Strong, 1958). Helixin (Leben et al., 1952) is probably identical with endomycin (Waksman and Lechevalier, 1953).

Reviews of actual and potential uses for these and other antibiotics in plant disease control have been presented by Anderson and Gottlieb (1952), Brian (1954), and Leben and Keitt (1954). Earlier reviews of antifungal antibiotics by Reilly et al. (1945) and by Waksman et al. (1952) suffered from lack of material available for review. No recent and comprehensive review of the entire subject is presently available in the English literature.

The wide distribution of antagonistic microorganisms in nature has inspired some writers to speculate at length on the role of antibiotic production in ecology, particularly among soil populations. Most of these writers appear to be favorably inclined toward the opinion that antibiotic production occurs in such natural substrates as soils, and that the antibiotics produced there are important in fixing the outcome of the competitive struggle for survival among microorganisms in natural, mixed flora (Brian, 1949; idem, 1952; Jefferys, et al., 1953; Thornton and Skinner, 1953; Stallings, 1954; Skinner, 1956.) There is, however, considerable evidence against this idea, as has been pointed

out by Waksman, among others (Waksman, 1947). At any rate, it seems clear that if antibiotics are to be considered factors in soil ecology, the definition of the term antibiotic must be enlarged. Waksman, who first proposed the term, has defined "antibiotic" or "antibiotic substance" on several occasions (Waksman, 1947; idem, 1956).

The antifungal antibiotic which has perhaps commanded most attention to date is nystatin, first reported under the name fungicidin (Hazen and Brown, 1950; idem, 1951). This antibiotic was the first antifungal antibiotic to be produced commercially in large amounts and has found use clinically both alone and in combination with broad spectrum antibiotics. It is marketed by E. R. Squibb and Sons under the trade name "Mycostatin".

A large literature has accumulated about nystatin. This has included studies on its effect in experimental infections (Campbell et al., 1954a; idem, 1954b; Drouhet et al., 1956; Drouhet, 1955; Drouhet and Wilkinson, 1957; Brown et al., 1953; Hazen et al., 1953), chemistry (Dutcher et al., 1954), assay methods (Gold et al., 1953; Kramer, 1957), development of resistance (Littman et al., 1957), mode of action (Lampen et al., 1957; Bradley, 1958), and clinical reports too numerous to cite. Some of the information on nystatin has been summarized by its discoverers (Brown and Hazen, 1957). Much clinical information can be

found in the symposium on the therapy of fungus infections edited by Sternberg and Newcomer (1955).

A major contribution to our understanding of antifugal antibiotics was that of Oroshnik et al. (1955) who pointed out that a number of the then known antifungal antibiotics exhibited in the ultra-violet region absorption characteristics similar to pure polyenic compounds -- that is, compounds with conjugated carbon-to-carbon double bonds. These authors showed further that the number of such conjugated double bonds might be determined by the characteristic absorption peaks. Thus tetraenes give peaks at or near 292, 304, and 318 - 320 millimicrons; pentaenes give peaks at or near 318, 333, and 351 millimicrons; hexaenes give peaks at or near 337 - 341, 357, and 378 millimicrons; heptaenes give peaks at or near 360 - 364, 377 - 384, and 399 - 406 millimicrons.

Although it was previous known that highly unsaturated compounds may be found in natural products, particularly from plants (Cf. reviews of naturally occurring acetylenic compounds by Anchel, 1954, and by Bu 'Lock, 1956), the occurrence of polyenes in the metabolic products of streptomycetes had not previously been suspected. That these substances do indeed occur in many streptomycetes isolated from soil has now been well established (Pledger and Lechevalier, 1956; Ball, et al.,

1957; Vanek et al., 1958). Although no metabolic function is presently known for these compounds, there is some indication that they may be concerned with lipid metabolism. Thus oils and fatty acids added to fermentation media are said to increase markedly the yield of the polyenic antibiotics fungichromin (McCarthy et al., 1955) and filipin (Brock, 1956).

Gottlieb et al., (1958) have recently made the interesting observation that hexane extracts of carrots and soybeans reversed the inhibitory action of a number of polyenes against test fungi. Sterols such as cholesterol, ergosterol, sitosterol, and stigmasterol were able to substitute for the hexane extracts of carrot and soybean in this reversal. The authors have suggested that the polyenic antifungal agents act by affecting carotenoid synthesis in fungi. These results do not, of course, explain the function of the polyenes in the actinomycetes that produce them. However, it is of interest that so far there has been no report of a polyene antibiotic which inhibits the growth of a streptomycete.

Many antifungal antibiotics have been reported since the discovery of nystatin. Most of these have been found among the metabolic products of various streptomycetes, and many of them have been shown to be polyenes. No purpose would be served by an attempt to describe the properties

of each of these antibiotics. Their number is so large that it would be desirable now to have a system for classifying antifungal antibiotics. Any number of means might be employed for arranging such a classification. One possible scheme, based on source of the antibiotic (plant, mold, bacterium, streptomycete) and type of compound (polyenes and nonpolyenes) is suggested in Figure I.

An attempt has been made in Table I to list all the known antifungal antibiotics produced by actinomycetes. This list undoubtedly comprises only a small portion of the many antifungal antibiotics which have been studied. This is partly so because many experimental antibiotics never find their way to the scientific literature. Others have been described in inaccessible publications. The list given here, however, is suggestive of the large number of antifungal antibiotics from actinomycetes reported in the easily available literature between 1940 and early 1958.

It is not our purpose here to review the literature on the actinomycetes. However, since part of this investigation has been concerned with the characterization of what we believe to be a new species of the genus Streptomyces, citations to some of the pertinent literature will be made.

For many years, study of the streptomycetes was a labor of love among a few specialists. Perhaps no group

of organisms in the history of biology ever suffered such sudden notoriety as did this group when it was found that its members comprise a rich source for new antibiotics. For a period of fifteen or more years literally thousands of isolations from soil have been made daily. Thus the number of cultures accumulated so fast that the old taxonomic schemes could not support them. This rapid growth of interest in the streptomycetes is reflected in the increased number of species listed in the few taxonomic schemes that have attempted to keep pace with the growing list of new cultures. Krassilnikov (1941), in his Guide to the Ray Fungi, listed only forty-seven species, whereas Bergey's Manual, sixth edition, listed seventy-three. Waksman and Lechevalier (1953) listed 146 species, and the seventh edition of Bergey's Manual (Breed et al., 1957) listed 149. But even these rapidly expanding lists do not include most of the species named in the recent literature. It is so commonplace to find a streptomycete described as a new species, or to find it described simply as Streptomyces sp., that the species concept has -- temporarily at least— lost all meaning among the streptomycetes.

Attempts to solve this difficulty have led to suggestions that a "group" concept be employed among the streptomycetes. Burkholder et al. (1955) and Hesseltine et al. (1955) presented criteria for distinguishing

streptomycetes and suggested group or type concepts. Waksman later (1957) presented a vigorous case for the retention of the species concept. At the present time, however, it appears that most workers are inclined toward "lumping" rather than "splitting" among the streptomycetes. The classification presented by Pridham *et al*. (1958) is an example of recent attempts to bring some order into the genus by "lumping".

A. Not produced by streptomycetes

B. Produced by plants (tomatin,* etc.)

BB. Produced by molds (gliotoxin, etc.)

BBB. Produced by bacteria (fluvomycin, ** etc.)

AA. Produced by streptomycetes

C. Active against bacteria as well as fungi (actinomycin, etc.)

CC. Active chiefly against fungi

D. Not polyenes (cycloheximide, etc.)

DD. Polyenes

E. Tetraenes (nystatin, etc.)

EE. Pentaenes (filipin, etc.)

EEE. Hexaenes (fradicin, etc.)

EEEE. Heptaenes (amphotericin B, etc.)

* Irving, et. al, 1946

** Carvajal, 1953

Figure 1 A Scheme for Classifying Antifungal Antibiotics.

# TABLE I

## A LIST OF ANTIFUNGAL ANTIBIOTICS FROM STREPTOMYCETES

| |
|---|
| Actinomycin (Waksman and Woodruff, 1940) |
| Cycloheximide (Wiffen, 1942) |
| Antimycin A (Leben and Keitt, 1948) |
| Musarin (Arnstein et al., 1948) |
| Fradicin (Swart et al., 1950) |
| Endomycin (Gottlieb et al., 1951) |
| Nystatin (Hazen and Brown, 1951) |
| Nigericin (Harned et al., 1951) |
| Rimocidin (Davisson et al., 1951) |
| Ascosin (Hickey et al., 1952) |
| Helixin (Leben et al., 1952) |
| Chromin (Takahasi and Tsubura, 1952) |
| Thiolutin (Seneca et al., 1952) |
| Antimycoin (Raubitschek et al., 1952) |
| Trichomycin (Hosoya et al., 1952a, 1952 b) |
| Moldin (Maeda et al., 1952) |
| Pleocidin (Charney et al., 1952) |
| Cacomycetin (Wakiki et al., 1952) |
| Mycelin (Aiso et al., 1952) |
| Phaeofacin (Maeda et al., 1952) |

TABLE I

A LIST OF ANTIFUNGAL ANTIBIOTICS
FROM STREPTOMYCETES (CONTINUED)

Ratoventin (Hosoya et al., 1952a)

Candicidin (Lechevalier et al., 1953)

Flaveolin (Takahashi, 1953a)

Flavacid (Takahashi, 1953b)

Oligomycin (Smith et al., 1954)

Candidin (Taber et al., 1954)

Candimycin (Shibata et al., 1954)

Sistomycosin (U.S. patent 712,547, 1954)

Mycolutein (Schmitz and Woodside, 1955)

Anisomycin (Tanner et al., 1955)

Fungichromin (Tytell et al., 1955)

Filipin (Ammann et al., 1955)

Amphotericins A and B (Steinberg et al., 1956)

Eulicin (Charney et al., 1956)

Antibiotic 1968 (Oswald et al., 1956)

Mycothricin (Rangaswami et al., 1956)

7,071R.P. (Despois et al., 1956)

Flavensomycin (Craveri and Giolitti, 1957)

Amidomycin (Vining and Taber, 1957a, 1957b)

Flavofungin (Uri and Bekesi, 1958)

TABLE I

A LIST OF ANTIFUNGAL ANTIBIOTICS

FROM STREPTOMYCETES (CONTINUED)

PA 150, PA 153, and PA 166 (English and McBride, 1958)

Pimaricin (Struyk et al., 1958)

Tennecetin (Burns and Holtman, 1959)

# CHAPTER III

## METHODS AND RESULTS

> I know that the tune I am piping is a very mild one (although there are some terrific chapters coming presently).....
>
> W.M. Thackery, in "Vanity Fair"

### A. Isolation and Characterization of the Antibiotic-Producing Organism

#### Isolation of the Organism

*Streptomyces chattanoogensis*, the antibiotic-producing organism used throughout this study, was isolated from soil in Chattanooga, Tennessee. The soil sample was representative of the characteristic red clay found in the Chattanooga area. The organism was isolated on nutrient agar containing 3 per cent glycerol without the addition of bacterial inhibitors.

#### Maintenance of the Organism

The organism was carried by weekly or bi-weekly transfers on slants of 3 per cent glycerol agar; later Carvajal's oatmeal agar (Pridham, *et al.*, 1957) was used for this purpose.

Stock cultures were kept at room temperature and in the refrigerator. Viability appeared to be much

better at room temperature, however; therefore most of the experiments reported here were performed with cultures that had remained at room temperature through many transfers. Attempts to maintain the organism in sterile soil were not successful. On the other hand, well-sporulated cultures on glycerol agar, stored at -20°C remained viable for more than a year.

## Macroscopic morphology

The organism was isolated, carried in subcultures, and used for antibiotic production for approximately eight months without exhibiting sporulation that could be recognized macroscopically. It was not until transfers were made to oatmeal agar that good sporulation was observed. Subsequently, other media, such as starch-inorganic salts agar (Pridham *et al.*, 1957) and potato infusion agar (Difco), were found to support excellent sporulation. However, during early experience exclusively with the vegetative stage of growth it became possible to recognize the culture by its distinctive colonial morphology in the vegetative stage. The following description is taken from observations made on isolated colonies on glycerol-phytone-yeast extract agar (*vide infra*) following incubation for six days at room temperature:

No aerial mycelium is present. The color of the

vegetative mycelium is cream to tan. The diameter of the isolated colony is 3 - 4 millimeters. (Well isolated colonies may attain a diameter of up to 15 millimeters within four weeks.) Close inspection of the colony shows it to be composed of two parts: a serrated or papillate lower portion extending about three-fourths of a millimeter above the surface of the agar, and a smooth, dome-shaped upper portion extending another three-fourths of a millimeter above the agar. Viewed from above, the colony is seen to be composed of a smooth central portion with papillae extending radially to the edge of the colony, giving the edge an erose or dentate appearance. The colony is tough and leathery and extends into the agar; when colonies are lifted in toto and freed from adhering agar, the underside is seen to be hollowed out with a concave depression, similar to the underside of an umbrella.

Figures 2a and 2b show sketches of this typical colonial appearance on GPY agar. Figures 3 and 4 show color photographs of the organism on several media.

## Pigment Production

One of the most consistent and characteristic properties of Streptomyces chattanoogensis is its ability to produce a diffusible yellow or yellow-orange pigment in most media. The pigment appears to be produced best

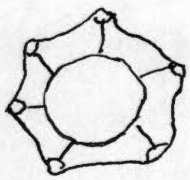
on media which best support sporulation of the organism. Furthermore, we have repeatedly observed that in shake flask experiments the early development of a deep yellow color invariably accompanies good antibiotic yeilds. Despite this apparent relationship, and despite the fact that the antibiotically active crude solids derived as the end product are yellow-brown in color, the pigment and the antibiotic are different and distinct substances, as will be shown later.

Solubility of the pigment was determined by flooding agar cultures with various solvents and, after a period of a few minutes, pouring off the solvent and examining visually for pigment. Using this method, it was found that the pigment was soluble in the following solvents: distilled water, methanol, ethanol, n-propanol, isopropanol, n-butanol, acetone, glycerol, 2-4 lutidine, and 2-4-6 collidine. Solvents in which the pigment was found not to be soluble by this method were: n-amyl alcohol, isoamyl alcohol, ether, petroleum ether (b.p. 30 - 60 C), ethyl acetate, amyl acetate, hexane, chloroform, tetrachloroethane, xylene, dioxane, and carbon tetrachloride.

## Growth of *Streptomyces chattanoogensis* on Various Media

*Streptomyces chattanoogensis* has been grown on many different agar media, but there is no advantage in describing

a.



b.

Figure 2

a. Sketch of typical isolated colony of Streptomyces chattanoogensis viewed from the side.

b. Same, viewed from above.

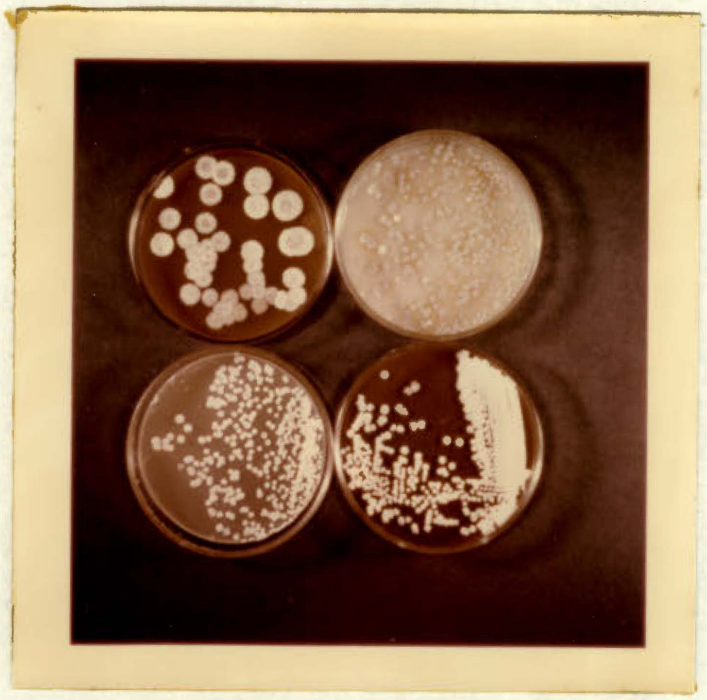


Figure 3

Appearance of *Streptomyces chattanoogensis* on Various Agar Media

Upper left: glycerol agar, 23 days

Upper right: Inorganic salts - starch agar, 14 days

Lower left: Phytone agar, 7 days

Lower right: Czapek-Dox agar, 14 days

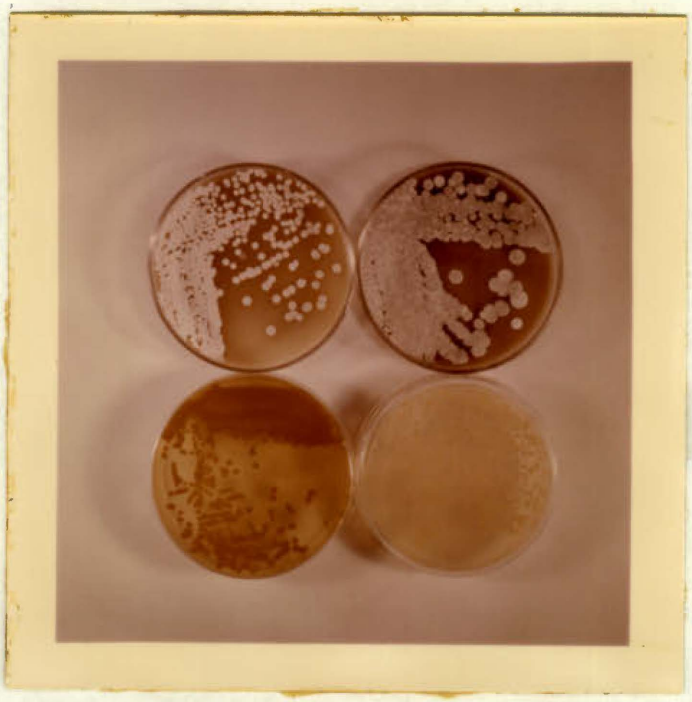


Figure 4

Appearance of *Streptomyces chattanoogensis* on Various Agar Media

Upper left: Oatmeal agar, 7 days

Upper right: Oatmeal agar, 24 days

Lower left: Czapek-Dox agar, 14 days (reverse)

Lower right: Phytone agar, 7 days (reverse)

morphology on each of these media. Many, such as nutrient agar and corn meal agar, did not support good growth. In general,media containing starch permitted most vigorous growth. On the other hand, simple media such as glucose-asparagine agar (Waksman, 1950) often gave good growth. The addition of yeast extract or malt extract to agar media usually favored more rapid growth and better pigment formation. Sporulation and pigmentation occurred best on oatmeal agar and other starch-containing agar media. However, on potato plugs, growth was somewhat slow, sporulation did not occur, the yellow pigment was not produced, and at ten days the potato plug turned black.

Carvajal's oatmeal agar was selected as the medium for carrying stock cultures of Streptomyces chattanoogensis because the organism always grew well, sporulated abundantly, and in general behaved predictably on this medium. Mass inoculation, usually be means of a moistened cotton swab, was used throughout in order to avoid genetic variation in the culture. Under these conditions the yellow pigment was usually clearly evident within twenty-four hours, and often before growth was clearly evident macroscopically. Good growth of vegetative mycelium appeared in two to three days. Sporulation usually occurred within six days, sometimes within four days. The spore coat was at first white. Later (after about ten to fourteen days) it turned

gray. The diffusible pigment, which was at first bright yellow, turned a deeper color, almost orange, as the spore coat became darker. In old cultures (three weeks or more at room temperature) the pigment was almost brown.

Physiological Characteristics

The following physiological characteristics of Streptomyces chattanoogensis were ascertained by repeated tests using the usual bacteriological methods as applied to the study of streptomycetes:

Starch is rapidly hydrolyzed.

Gelatin is liquified.

Blood is hemolyzed.

The organism is catalase positive.

When tested according to the method of Jeffries et al. (1958) the organism hydrolyzes deoxyribonucleic acid.

Hydrogen sulfide is not produced on peptone-iron agar.

Nitrates are reduced.

Milk is coagulated and peptonized. Occasionally tubes of litmust milk showed a heavy ring of yellow growth at the surface of the medium. However, a true pellicle covering the surface was never observed in any liquid medium.

Growth without sporulation occurs at 37°C but not at 45°C.

Growth is inhibited in media containing 3 per cent sodium chloride.

Agar cultures give off the typical musty odor of streptomycetes.

In shake flask cultures a distinct odor is produced. The odor is faint, but quite characteristic, and unlike that of any other culture in our experience.

## Microscopic Morphology

Microscopic morphology was studied chiefly by the impression slide method essentially as recommended by Drechler (1919) but without permanent staining and mounting. We have also made extensive use of a "Scotch tape" modification of this method, in which a piece of scotch tape is pressed, sticky side down, against a well-developed colony and then dipped briefly into a one per cent aqueous solution of crystal violet. The excess stain is drained against a piece of blotting paper and the scotch tape spread firmly over a clean glass slide. When such preparations are properly made, many microscopic fields showing morphological structures typical of streptomycetes may be found.

All observations of microscopic morphology of

Streptomyces chattanoogensis were made from cultures grown at least one week on the surface of Carvajal oatmeal agar or on starch-inorganic salts agar. Figure 5 is a photomicrograph of an impression smear made by the scotch tape method. It shows the typical spirals and chains of spores found in such preparations.

The loosely coiled sporophores are abundant in well-developed cultures. Characteristically, these sporophores bear long chains of conidiospores. The conidiospores measure 1.1 to 1.3 microns in diameter and appear to be very nearly spherical. Although they may be found free and at the sides of hyphae, their predominant position is at the ends of the coiled sporophores from which they arise.

The vegetative mycelium of Streptomyces chattanoogensis is not remarkable. It is approximately one micron in diameter; this diameter is quite uniform, the organism not being prone to develop swellings and other irregular forms on the media used.

The organism is gram positive and not acid-fast.

## Taxonomic Considerations

Comparisons of our culture with authentic strains of other yellow-pigmented streptomycetes and with descriptions in the literature have not permitted identification of this organism as a previously described species of

Figure 5

Photomicrograph of Scotch Tape Impression Smear of Streptomyces chattanoogensis

Streptomyces. Thus, Streptomyces chattanoogensis differs from Streptomyces aureus ATCC 3309 in that the latter does not produce a white-gray spore coat. The spores of Streptomyces flaveolus ATCC 3319 were measured and found to be 0.9 to 1.0 microns in diameter and are somewhat cylindrical, whereas the spores of Streptomyces chattanoogensis measured 1.1 to 1.3 microns in diameter and appeared to be perfectly spherical. Two strains of Streptomyces rimosus (NRRL B-2234 - Pfizer strain, and a strain from Purdue University) gave dry, wrinkled growth and were not easily induced to sporulate. This organism is also described as producing cylindrical spores, sporulating poorly on starch agar, and hydrolyzing starch only slightly (U. S. patient 719,878, 1954). After some experience with these cultures, S. aureus, S. rimosus, S. flaveolus, and S. cellulosae could be recognized by gross appearance and could be distinguished from each other and from Streptomyces chattanoogensis on this basis. Furthermore, none of these strains produced tennecetin in GYP medium, nor did they present the typical colonial morphology already described for Streptomyces chattanoogensis.

Using the system of Waksman and Lechevalier (1953), the organism may be placed in the Streptomyces flavus group. Or, it may be placed in Group VIa of the earlier grouping of workers at the Northern Regional Research.

Laboratory (Hesseltine et al., 1954) or in Section Spira, gray series, of the same workers' later classification (Pridham et al., 1957).

## B. Microbiological Assay Method for Tennecetin

In studying any antibiotic it is essential that a reliable assay method be available. When the antibiotic under study is a new one, a method must be devised specifically for that antibiotic. A larger portion of this investigation was therefore concerned with designing and testing a microbiological assay method for tennecetin.

Very early in the course of these studies it was evident that tennecetin diffuses readily through agar and lends itself well to an agar plate diffusion assay method. At first a strain of Hansenula anomola was used as the test organism. However, it appeared that almost any yeast or mold might be used, and for reasons of convenience, a strain of Saccharomyces carlsbergensis, with which we were familiar from other work, was chosen as the standard test organism. This strain, received from Dr. J. Orvin Mundt, is referred to in our collection as Saccharomyces carlsbergensis K-20, and is probably identical with NRRL Y-1025. It has been used as the assay organism throughout the studies reported here, and is the strain supplied by us to other laboratories for assaying tennecetin.

The protocol for the assay method eventually devised and adopted for measuring amounts of tennecetin in broths and solutions of solids is as follows:

Materials

Test organism: Saccharomyces carlsbergensis, K-20.

Base agar: 2 per cent agar without nutriments.

Inoculum broth: Bacto-peptone, 0.5 per cent; glucose, 1.0 per cent; Difco yeast extract, 0.3 per cent; tap water.

Overlay agar: Inoculum broth plus 1.0 per cent agar.

Maintenance agar: Inoculum broth plus 1.5 per cent agar, put up as slants in tubes.

Paper discs: Schleicher and Schuell #740-E, 12.7 millimeters diameter.

Plates: Standard Petri dishes, 10 centimeters diameter.

Technique

Plates are poured with 20 ml. base agar and allowed to harden. Such plates maybe prepared at intervals and stored in the refrigerator until needed. They should be brought to room temperature before use.

The test organism is maintained by weekly transfer on maintenance agar. Transfers from maintenance agar to

inoculum broth are used for the test proper. The inoculum for each day's assay is made from an 18 to 24 hour culture in inoculum broth. (All incubations in this assay are at room temperature.)

Overlay agar is inoculated at a rate of one milliliter inoculum broth culture per 100 ml. overlay agar. Overlay agar is conveniently prepared in 100 ml. quantities in flasks and is melted and cooled before use.

Ten milliliters of inoculated overlay agar is poured and evenly distributed over the base agar. The overlay agar should be allowed to harden for thirty minutes before the discs are placed on its surface. The placing of discs should be done within two hours of pouring the inoculated overlay agar (vide infra).

Samples to be assayed are diluted to contain approximately 2.0 units per ml. The test solutions are assayed by dipping a paper disc into the solution until it is saturated, allowing the disc to drain against a glass surface (e.g., the wall of the vessel in which the dilutions are contained), and placing the disc on the surface of the overlay agar. No more than five discs should be placed on one plate.

The results of the assay are determined after 18 to 24 hours incubation at room temperature by measuring the diameter of the zones of inhibition (measuring across

the center of the disc). Zone edges are always very sharp, and in properly performed assays the zones should be very nearly perfect circles. Precision in reading, of course, depends upon the precision of the device used for measuring zones.

## Calculations

Calibrating a standard solution:

As crystalline tennecetin is not yet available, and potency is therefore not standardized on a weight basis, tennecetin is measured in terms of units of activity. A unit of tennecetin has been arbitrarily defined as follows: One unit of tennecetin is that amount of antibiotic which, when contained in 1.0 ml. water, will give a zone of exactly 20.0 mm. diameter when assayed by the standard plate-disc method. Thus, the potency of any solid or liquid sample is equal to the dilution factor required to give a 20.0 mm. zone by the standard assay. A gram of solids, dissolved in 100 ml. water, and further diluted 1:100 with water, and this dilution giving a 20.0 mm. zone by the standard assay, would give an assay value for the original solid of 10 units per milligram. A liter of crude broth, diluted 1:200 with water, and this dilution giving a 20.0 mm. zone, would assay 200 units per milliliter.

It has been found that tennecetin dissolved in water gives a logarithmic dose-response curve over a ten-fold change in concentration by the standard assay method. Thus, 10 units per ml. give a zone of 30 mm. Furthermore, dilutions giving between one and ten units per milliliter fall on a straight line when mm. zone diameter is plotted against logarithm of concentration in units per milliliter. Thus, to prepare a standard curve, it is convenient to use a solution which gives, undiluted, a 30 mm. zone (hence, contains 10 units per ml.). Such a solution can be diluted by tenths to give points on the curve of 9, 8, 7, 6, 5..... 1 unit per ml.

Figure 6 shows a standard curve plotted in the manner described. This curve has been repeated many times. All points fit the curve well, and the slope (increase in units on abcissa per doubling of units on ordinate) remains remarkably constant at 3.0 from day to day.

At concentrations below 1.0 unit per ml. and above 10 units per ml. the curve begins to flatten, and should not be extrapolated or interpolated. When samples do not fall on the curve, the assay should be repeated at other dilutions. Best results are obtained when samples are diluted to an estimated 2.0 units per ml.

It should be emphasized that tennecetin preparations

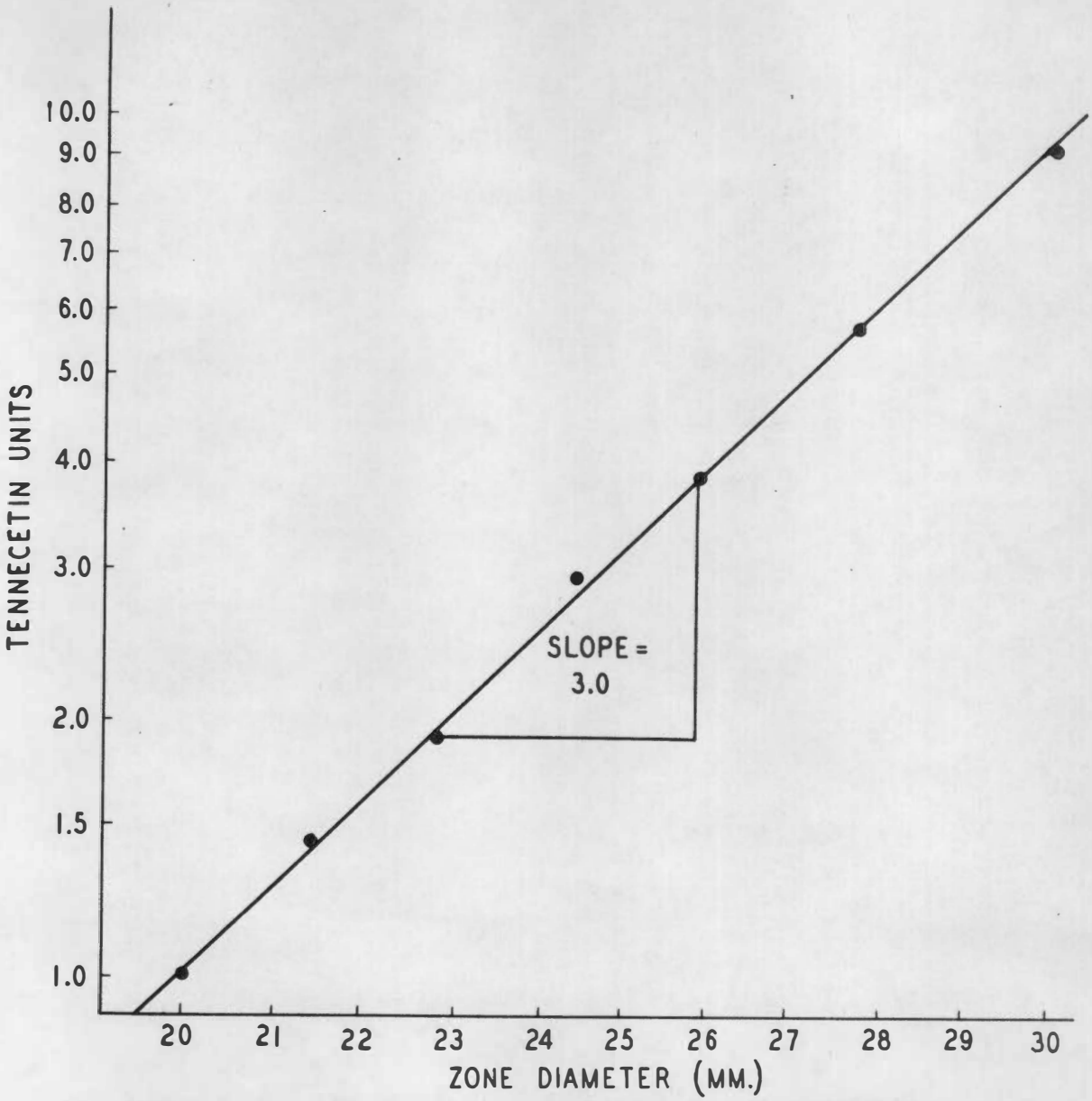



Figure 6. Standard Assay Curve for Tennecetin

have been assayed in terms of antibiotic activity. The unit of activity is an arbitrarily defined amount as determined by the standard plate assay method defined above. Thus, tennecetin preparations have not been assayed by reference to a standard preparation. However, it is possible to prepare solutions of known activity, and to include them in the assay procedure to keep under surveillance variation due to the technique of the test itself, and this was done in most of our assays. But it must be understood that since pure crystalline tennecetin is not yet available, and since the stability of solutions and dried preparations is not definitely known, the master standard is the test itself, and all preparations are assayed in terms of performance under the conditions of the test alone, not in reference to performance relative to that of a crystalline master standard.

Experiments on Assay Design

The importance of the time interval between pouring assay plates and positioning impregnated discs was shown in an experiment, the results of which are recorded in Table II. In this experiment, the same solution of tennecetin, diluted to contain approximately 2.0 units per ml., was assayed by the standard method except that the time interval mentioned was varied between five

## TABLE II

### EFFECT ON DIAMETERS OF INHIBITION ZONES OF VARIOUS TIME INTERVALS BETWEEN INOCULATING ASSAY PLATES AND POSITIONING DISCS

| Time Interval (min.) | Zone Sizes (mm.) | Average Zone Size (mm.) | Units per ml. |
|---|---|---|---|
| 5 | 24.5, 24.5, 24.0 | 24.3 | 2.65 |
| 40 | 24.0, 24.0, 24.0 | 24.0 | 2.50 |
| 60 | 24.0, 23.5, 23.5 | 23.8 | 2.35 |
| 120 | 24.0, 23.5, 23.5 | 23.6 | 2.25 |
| 160 | 23.0, 23.5, 22.0 | 23.0 | 2.00 |
| 270 | 22.0, 21.5, 22.0 | 21.8 | 1.50 |

minutes (immediately after the overlay agar had hardened) and 270 minutes. As can be seen, assay results become smaller as the interval is lengthened. This is probably due to the fact that the agar gel becomes progressively firmer during the first hour or so, thereby impeding diffusion, and also to the fact that the test organism is beginning to multiply at about the same time. Evidently inhibition zones are larger when fewer organisms are present. This was confirmed by a similar experiment in which the amount of inoculum was varied between 1.0 and 5.0 per cent. In this experiment, large inocula gave smaller inhibition zones than did smaller inocula when tested against the same antibiotic solution.

Table III and Figure 7 show the results of a typical assay. In this experiment, tennecetin solids obtained from Merck and Company, estimated purity 6.5 units per milligram, were used. One hundred and ninety-one milligrams of the material were dissolved in fifty milliliters of water to give 3.82 mg. per ml., or approximately 25 units per ml. This solution was therefore diluted 1:2.5 in water to give an estimated 10.0 units per ml. Further dilutions were then made to give assay samples of estimated potency of 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 (no dilution) units per ml.

In Table III values for each of three assay discs

## TABLE III

### TYPICAL TENNECETIN ASSAY DATA SUITABLE FOR CONSTRUCTION OF STANDARD CURVE

| Dilution | Zone Sizes (mm.) | Average Zone Size | Units (*) | Assay Value (u./ml.) |
|---|---|---|---|---|
| 1:10 | 20.5, 21.5, 21.0 | 21.0 | 1.25 | 12.5 |
| 2:10 | 23.5, 24.0, 24.0 | 23.7 | 2.30 | 11.5 |
| 3:10 | 25.0, 25.5, 25.0 | 25.3 | 3.40 | 11.3 |
| 4:10 | 27.0, 27.0, 26.5 | 26.7 | 4.60 | 11.5 |
| 5:10 | 28.0, 28.0, 28.0 | 28.0 | 6.30 | 12.6 |
| 6:10 | 28.5, 28.0, 29.0 | 28.5 | 7.00 | 11.7 |
| 7:10 | 28.5, 28.5, 29.0 | 28.8 | 7.50 | 10.7 |
| 8:10 | 29.0, 29.5, 29.5 | 29.3 | 8.50 | 10.6 |
| 9:10 | 29.5, 30.5, 31.0 | 30.3 | (10.1) | (11.2) |
| str. | 31.0, 31.0, 31.0, | 31.0 | (10.25) | (10.3) |
| | | | Average: | 11.55 u/ml. |

(*) The values in this column refer to readings taken from a previously prepared standard curve. The values in the last column were obtained by multiplying this value by the dilution factor.

( ) Values in parentheses are off the standard curve and were not included in calculating the average.

are included to show the normal variation between reading of the same dilution on different discs. When values for the eight dilutions falling on the standard curve are averaged, the average assay value is 11.55 units per ml. This is equivalent to a value of 7.3 units per milligram of the original material.

Figure 7 shows that when the average zone sizes obtained for each of these dilutions are plotted in the usual manner, a straight line results, and this line is parallel to the slope of the standard curve.

Repeated assays on the same material have indicated that the error of the standard assay method is somewhat less than ± 10 per cent.

C. Methods for Producing Tennecetin

Surface Culture

Extraction of cultures of Streptomyces chattanoogensis grown on agar media represented a convenient method for producing small amounts of tennecetin. In this method, plates of a suitable agar medium such as GPY medium containing 1.5 per cent agar were heavily seeded by surface inoculation with a suspension of spores or mycelium from a vigorous young culture. After four or five days incubation at room temperature the cultures were extracted by steeping in water or a water-miscilble solvent such as

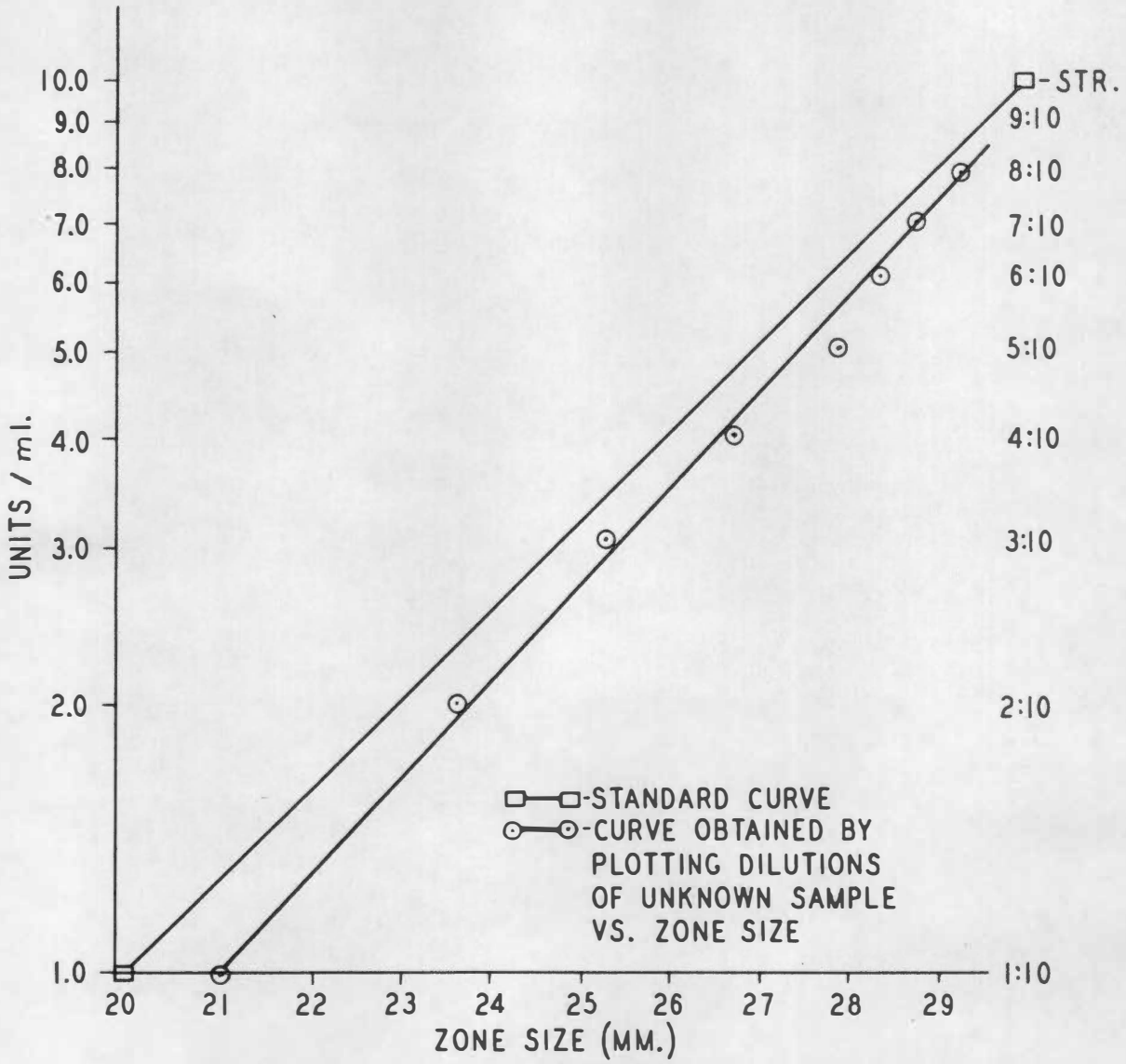



Figure 7. Curve obtained when different dilutions of the same tennecetin solution were plotted against zone sizes obtained on standard assay plates.
(The theoretical standard curve is shown for comparison.)

acetone. Several extractions were necessary to obtain all the activity from the cultures.

The resulting bright yellow extracts were combined and concentrated by evaporation. Purification of these extracts was achieved by fractional precipitation from butanol with ether, or the extracts were used without further purification.

Shake Flask Cultures

Most of the experiments reported here have been performed with material prepared from shake flask cultures. In this method, cotton-stoppered 500 ml. Erlenmeyer flasks containing 100 ml. liquid medium were usually used. Inoculation was made directly with spores or by the more reliable, so-called "submerged inoculation" -- i.e., with growth from a previous shaken culture. A good inoculum was found to be 2 per cent by volume of growth from a three to five day old shaken culture.

Most of these cultures have been incubated on a reciprocal shaking machine, although a rotary shaker has also been successfully employed. The shaking device used in most of the experiments reported here had a 3.0 inch stroke and a constant rate of 115 strokes per minute.

The optimal temperature for tennecetin production appeared to lie near 25°C. Lack of facilities for temperature control prevented an exact determination of the

influence of temperature on the fermentation. Except for a number of experiments conducted in an air-conditioned room with temperature controlled at 23°C, all work was done at "room temperature". This temperature, of course, varied throughout the year. During some summer months when room temperature remained near or above 30°C, tennecetin yields were very low.

Shake flask fermentations were studied by determining changes in pH, packed mycelial volume, (PMV), and tennecetin activity. For pH determinations, the glass electrode was used. Packed mycelial volume was measured by centrifuging forty milliliters of whole culture broth for 5 minutes at 1,000 rpm in graduated centrifuge tubes and noting the amount of growth packed in the bottom of the tube. The value was expressed as per cent. Tennecetin was measured in units according to the standard assay method, using the supernatant from the PMV determinations.

Laboratory Fermentors

Tennecetin has been produced in cultures in laboratory fermentors containing as much as eight liters of culture medium. For these studies two types of fermentors were used. One was constructed from the ordinary ten gallon laboratory glass "carboy". These cultures were aerated by forcing filtered air or oxygen through the

liquid medium and were agitated either by means of a motor-driven stirrer or by placing the carboy on a reciprocal shaking machine. The other type of laboratory fermentor used was of stainless steel construction, five gallons in capacity, equipped with a motor-driven stirring device.

Material from Other Sources

Some of the experiments reported upon here were performed with crude tennecetin obtained from and prepared by Merck and Company, Rahway, New Jersey, and by Charles Pfizer and Sons, Brooklyn, New York. These materials were prepared in pilot plant equipment and in large fermentors. Some lots of the Merck material were produced in ten thousand gallon production type fermentors. Details of the production methods used by these two companies are not known to us. Material from Merck and Company was a brownish-yellow powder assaying 7.3 units per milligram. Material from Charles Pfizer and Sons was finer in texture, lighter in color, and assayed 8.4 units per milligram.

## D. Recovery Procedure for Tennecetin

Filtration

When culture fluids of Streptomyces chattanoogensis were filtered through E & D #617 filter paper, clear

filtrates readily resulted. Almost all the antibiotic activity was found in the filtrate. Extraction of the filtered mycelium gave little more activity, and no more than did washing the mycelium in water. This, of course, may be a function of the medium used. However, it was so invariably true with cultures in GPY medium that the filtered cells were routinely discarded.

It was not necessary to acidify culture broths to facilitate filtration when C & D #617 filter paper was used. Ohter papers, such as Whatman #1, were much less satisfactory.

## Extraction

Tennecetin was extracted from culture filtrates with n-butanol. Two extractions were found to be sufficient to remove most of the activity from the aqueous phase. Butanol-water emulsions were broken by filtration through absorbent cotton. The optimal reaction for butanol extraction appeared to be slightly on the acid side of neutrality.

## Evaporation

Butanol extracts were best evaporated under vacuum at approximately 50°C. The volume was usually reduced to one-tenth or less of the original volume of the extract, but evaporation could not be carried completely to dryness

without considerable loss of antibiotic activity. This loss in activity was also accompanied by a dark brown colored product with a characteristic odor rather than the yellow, odorless active product.

Precipitation

Addition of four to five volumes of cold anhydrous ether to cooled butanol extracts readily precipitated tennecetin as a fine, light, yellow precipitate. Precipitation was usually complete within one hour at refrigerator temperature.

The precipitate was collected by centrifugation or by filtration through Whatman #1 paper.

Crude solids thus obtained have varied somewhat in color and in antibiotic activity. Light yellow solids had higher potencies than did darker, brownish solids. The highest yield obtained was approximately 200 units per mg. More often the yield was about 50 to 100 units per milligram. The usual yield in dry weight from shake flasks and laboratory fermentors was approximately forty milligrams per liter of original cuture medium. Thus, the usual yield in terms of units was approximately 2,000 to 4,000 units per liter.

## E. Experiments with Shake Flask Cultures

### Design of GPY Medium

Early experiments with shaken cultures utilized nutrient broth containing 2 per cent glycerol as the fermentation medium. Yields of tennecetin in this medium were never above two units per milliliter. Attempts were therefore made to design a better medium for tennecetin production in shaken cultures.

Table IV and Figure 8 show pH, PMV, and units of tennecetin activity for a typical shake flask experiment using the original nutrient broth - glycerol medium. (All values in these experiments are for pooled contents of triplicate shake flasks. All experiments were done on a reciprocal shaker with a 3.0 inch stroke, 115 strokes per minute, in a temperature-controlled room at 23°C.)

Table V and Figure 9 show the results obtained under the same conditions with the same medium supplemented with 0.2 per cent Difco yeast extract. (Subsequent experiments demonstrated that variations between 0.1 and 0.3 per cent yeast extract had little influence on the fermentation.)

Table VI and Figure 10 show the results obtained when 0.5 per cent phytone (Baltimore Biological Laboratories) was added to the nutrient broth - glycerol - yeast extract medium.

Table VII and Figure 11 show the results obtained in a typical experiment with the medium ultimately adopted for routine use and referred to as "GPY medium". In this experiment, determinations were again made on the contents of three pooled shake flasks (500 ml. flasks, 100 ml. medium per flask) by the methods already described. Inoculum consisted of 1 per cent by volume of a 38- hour shaken culture in GPY medium. The reciprocal shaker was again used. The only difference between this and the preceding experiment was that the fermentation was conducted at room temperature, which at the time of the experiment varied between approximately 25° and 29°C.

Further additions and substitutions in the formula failed to increase tennecetin yields. There was no observable difference in growth or yield of antibiotic when distilled water was substituted for tap water in the medium; hence tap water was used throughout. Addition of corn steep liquor decreased tennecetin yields and caused much foaming in the flasks. Addition of phosphate salts permitted good growth and rich yellow pigment production, but negligible yields of tennecetin.

The pH of autoclaved GPY medium is pH 7.0. Adjustment to pH 7.6 and addition of 0.25 per cent calcium carbonate prevented the rapid drop in pH characteristic of tennecetin fermentations in GPY medium, but did not

TABLE IV

CHANGES IN SHAKE FLASK CULTURES WITH NUTRIENT BROTH PLUS GLYCEROL

| Hours | PMV (%) | pH | Tennecetin (units) |
|---|---|---|---|
| 0 | 0 | 7.0 | 0.0 |
| 48 | 5.5 | 5.0 | 0.0 |
| 72 | 6.3 | 4.5 | 0.5 |
| 96 | 6.3 | 4.3 | 1.0 |
| 120 | 5.5 | 4.3 | 1.0 |

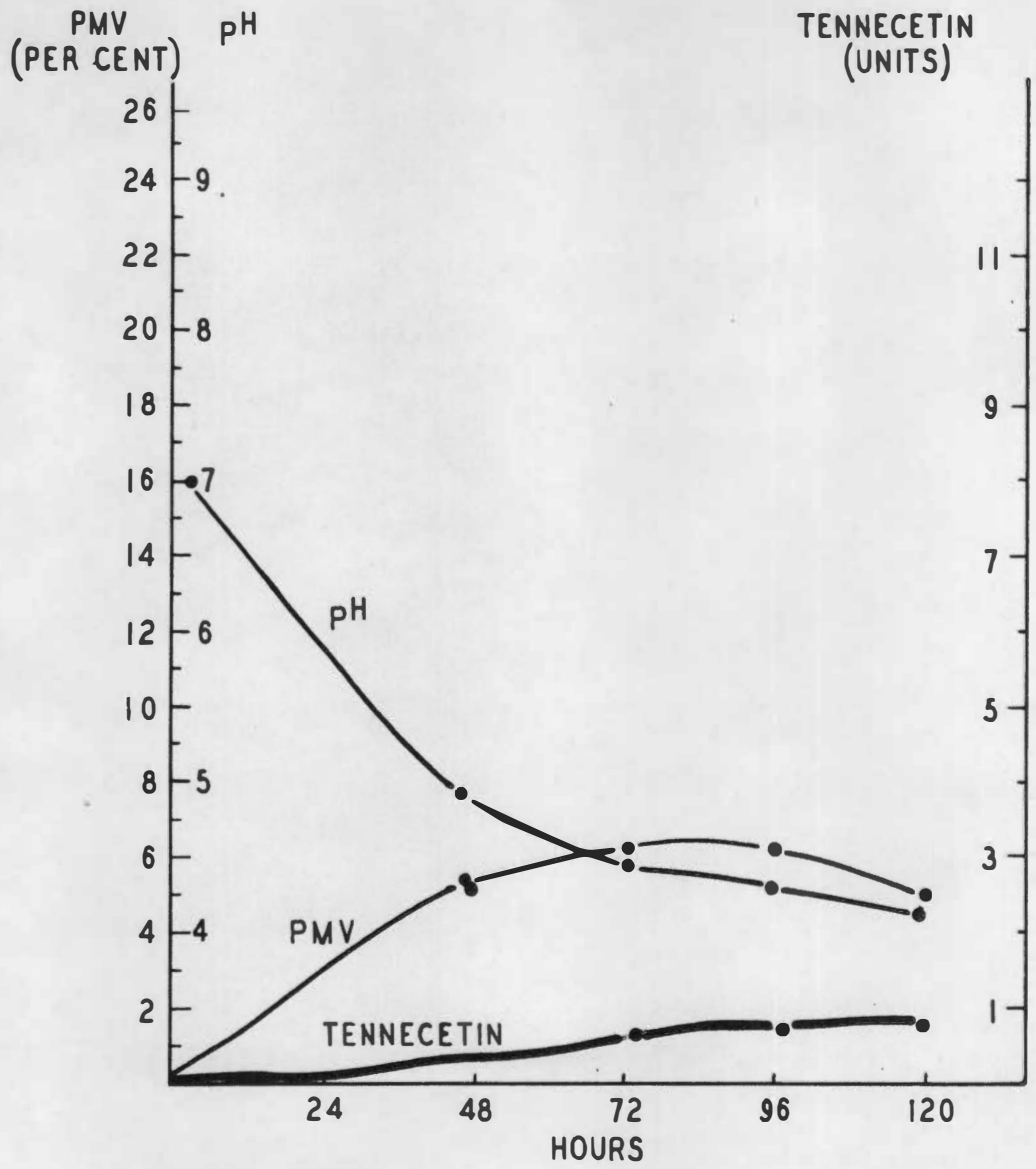



Figure 8. Changes in pH, PMV, and Antibiotic Content in Shake Flasks with Nutrient Broth Containing 2 per cent Glycerol.

TABLE V

CHANGES IN SHAKE FLASK CULTURES WITH NUTRIENT BROTH PLUS GLYCEROL PLUS YEAST EXTRACT

| Hours | PMV (%) | pH | Tennecetin (units) |
|---|---|---|---|
| 0 | 0.0 | 7.0 | 0.0 |
| 48 | 9.0 | 4.9 | 2.0 |
| 72 | 13.5 | 4.65 | 4.0 |
| 96 | 13.5 | 4.6 | 6.0 |
| 120 | 13.5 | 7.8 | 5.5 |

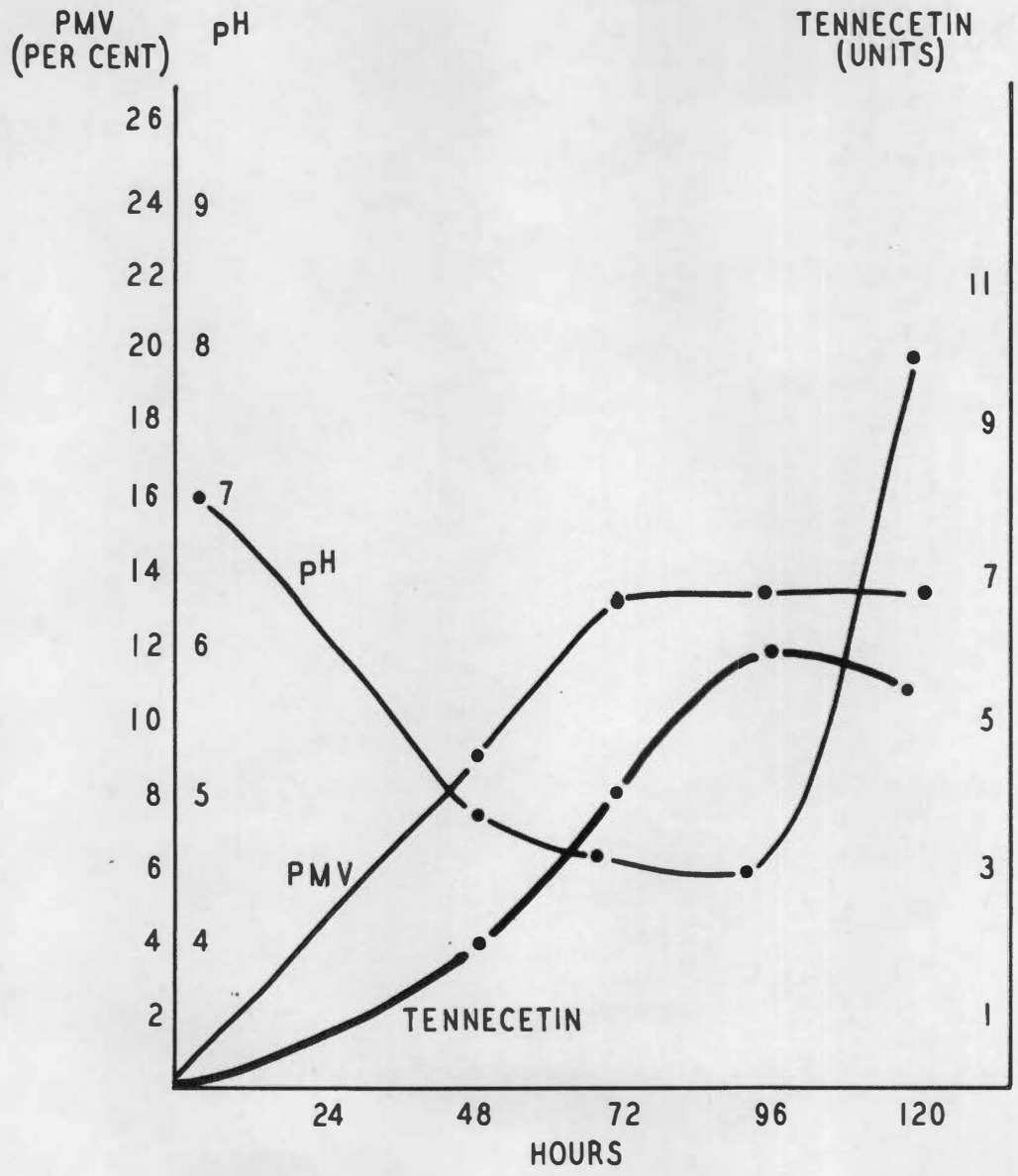



Figure 9. Changes in pH, PMV, and Antiotic Content in Shake Flasks with Nutrient Broth Containing Glycerol plus Yeast Extract.

TABLE VI

CHANGES IN SHAKE FLASK CULTURES
WITH NUTRIENT BROTH PLUS
GLYCEROL PLUS PHYTONE

| Hours | PMV (%) | pH | Tennecetin (units) |
|---|---|---|---|
| 0 | 0.0 | 6.9 | 0.0 |
| 48 | 13.2 | 5.3 | 3.0 |
| 72 | 16.2 | 4.4 | 4.0 |
| 96 | 22.0 | 4.7 | 6.0 |
| 120 | 23.5 | 8.1 | 10.0 |

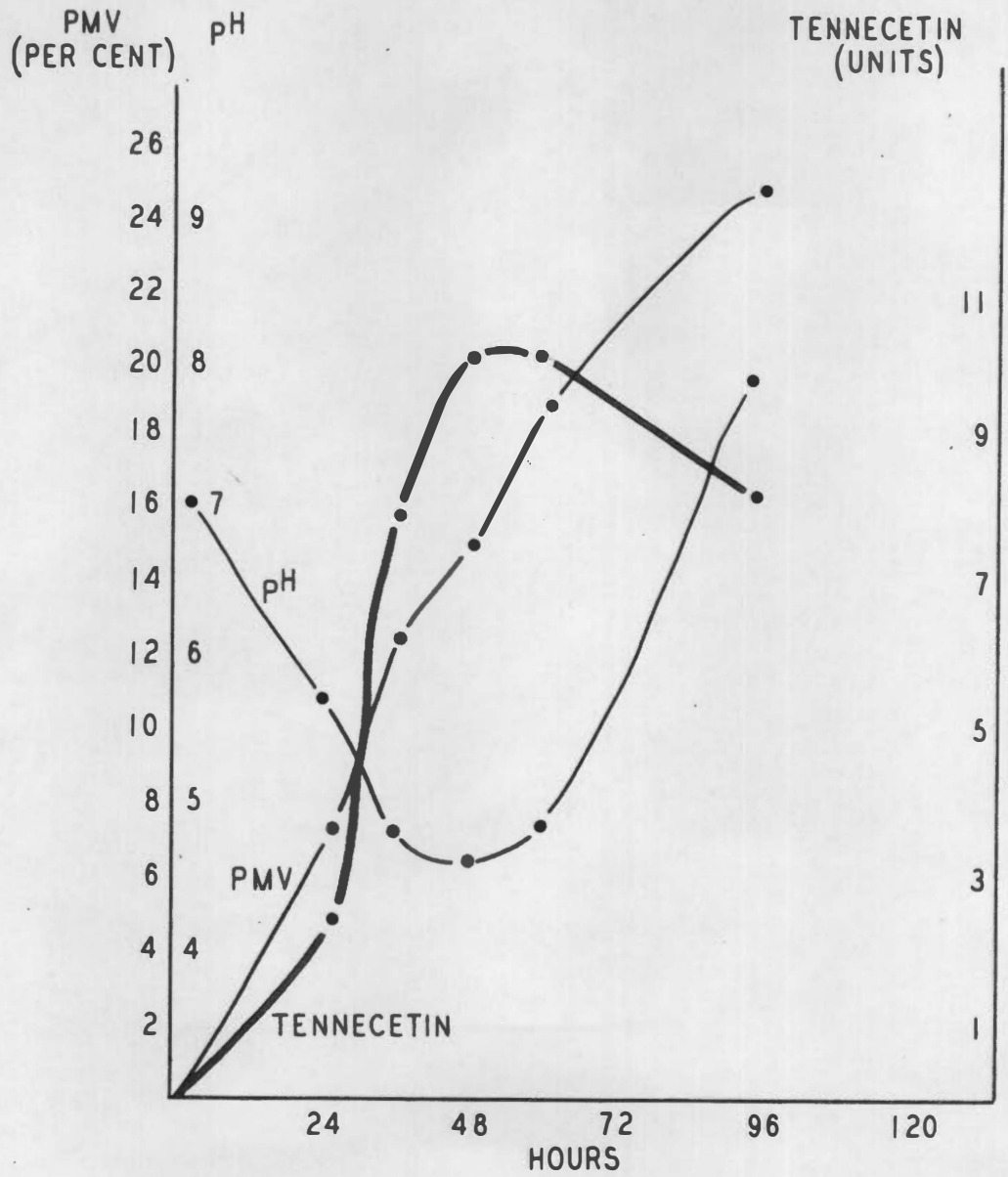



Figure 10. Changes in pH, PMV, and Antibiotic Content in Shake Flasks with Nutrient Broth Containing Glycerol, Yeast Extract, and Phytone.

TABLE VII

CHANGES IN pH, PMV, AND ANTIBIOTIC CONTENT IN A TYPICAL SHAKE FLASK EXPERIMENT USING GPY MEDIUM

| Hours | PMV (%) | pH | Tennecetin (units/ml.) |
|---|---|---|---|
| 0 | - | 7.0 | - |
| 48 | 14.0 | 5.3 | 3.0 |
| 72 | 16.5 | 4.5 | 3.5 |
| 96 | 22.0 | 4.8 | 5.8 |
| 120 | 24.0 | 8.1 | 10.0 |

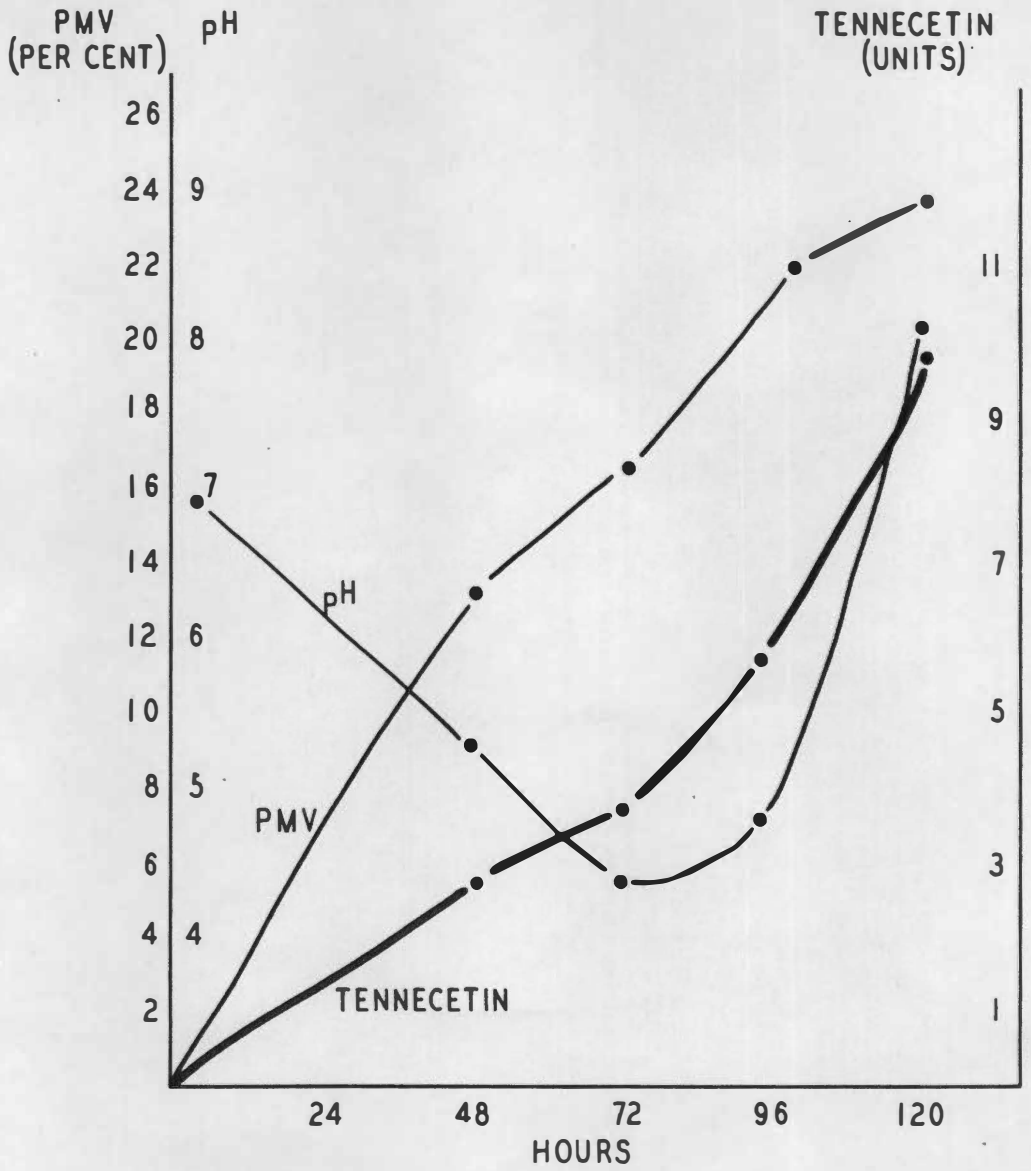



Figure 11. Changes in pH, PMV, and Antibiotic content in a Typical Shake Flask Experiment Using GPY Medium.

influence markedly the final yield of antibiotic (Table VIII).

Shake flask experiments in which the carbohydrate source in GPY medium was varied gave the following results in terms of tennecetin production: Dextrin, galactose, and inositol gave yields equal to or slightly higher than did glycerol. Glucose gave rapid growth but somewhat lower yields than did glycerol. Maltose gave lower yields than glycerol. Lactose gave very low yields (less than one unit of tennecetin activity per milliliter). No tennecetin was produced when sucrose was substituted for glycerol. In other experiments, in which nutrient broth was used as the basal medium, mannose, xylose, mannitol, inulin, and sorbitol failed to support tennecetin synthesis.

The results of these experiments and considerations of economy determined the final composition of the medium adopted for routine use and referred to as GPY medium. This medium has the following composition:

| | |
|---|---|
| Peptone (Difco) | 0.5 per cent |
| Beef extract (Difco) | 0.3 per cent |
| Yeast extract (Difco) | 0.2 per cent |
| Phytone (BBL) | 0.5 per cent |

Tap water

TABLE VIII

CHANGES IN SHAKE FLASK CULTURES
CONTAINING GPY MEDIUM WITH
AND WITHOUT CALCIUM CARBONATE

| Hours | Without $CaCO_3$ | | | With 0.25% $CaCo_3$ | | |
|---|---|---|---|---|---|---|
| | PMV (%) | pH | Tenn. (u./ml.) | PMV (%) | pH | Tenn. (u./ml.) |
| 0 | - | 7.6 | - | - | 7.6 | - |
| 27 | 30 | 5.8 | 7.0 | 30 | 6.85 | 9.0 |
| 36 | 30 | 5.25 | 9.0 | 35 | 6.6 | 13.0 |
| 51 | 48 | 7.65 | 10.0 | 48 | 7.6 | 10.0 |
| 60 | 45 | 7.9 | 10.0 | 53 | 7.9 | 12.0 |
| 78 | 52 | 8.1 | - | 53 | 8.25 | - |

## Attempts to Improve GPY Medium with Further Additions

The addition of oils and fatty acids to the culture medium apparently increases the yield of heptaene antibiotics. Using a spectrophotometric assay method, McCarthy *et al.* (1954) reported increases up to twenty times in fungichromin production when such substances as soy bean oil, oleic acid, Spans, and beta-carotene were added at 1 per cent levels to a glucose medium. Brock (1956), also assaying spectrophotometrically, reported yields of 2,000 to 4,000 micrograms per milliliter of filipin when lecithin, sperm oil, lard oil, methyl oleate, methyl myristate, methyl palmitate, hexadeconol, palmitic acid, triolein, ethyl palmitate, and 12-hydroxystearic acid were added at 2 per cent levels to 3 per cent soy flour medium.

However, additions of oleic acid, cottonseed oil, soy bean oil, Tween 80, and high concentrations of glycerol (up to 10 per cent) did not favorably affect tennecetin synthesis. Mevalonic acid, reported to increase yields of the tetraene antifungal antibiotic antimycoin (Schaeffer *et al.*, 1958) did not give increased yields of tennecetin when added to GPY medium at levels of 0.4 mg. per ml.

## F. Attempts to Increase Tennecetin Yields by Strain Selection

Colonies recovered from ultra-violet irradiated suspensions of spores and mycelium of Streptomyces chattanoogensis were tested in shake flasks for their ability to produce increased amounts of tennecetin. In no case was a strain found that produced yields higher than those of the non-irradiated parent strain.

Similar results were obtained when colonies were picked at random, grown in shake flasks, and their filtrates tested by the standard assay method.

Transfers of the parent culture of Streptomyces chattanoogensis were always made by mass inoculation of spores to guard against genetic variation in the culture. That such variation is considerable in species of Streptomyces is well known (Cf., for example, Carvajal, 1946; Jones, 1946; Williams and McCoy, 1953). That this is true also of Streptomyces chattanoogensis is shown by the following experiment.

A dilute spore suspension of the parent organism was spread over the surface of four plates of assay overlay agar. After ten days' incubation at room temperature, the plates were examined for numbers and types of colonies. Three types were recognized: well sporulated ("S colonies"); non-sporulated ("A colonies"); and mixed types, usually

showing sectoring of sporogenous and asporogenous growth ("M colonies").

Table IX gives the frequency of each of the three types found in this experiment.

Five colonies of each type were isolated in pure culture and tested in GPY medium in shake flasks for antibiotic production. Table X shows the type of growth and amount of antibiotic produced by each strain at the end of seventy-four hours in GPY medium. The table also gives an indication of the amount of growth of each strain at seventy-four hours under the heading SMV. This value (settled mycelial volume) was determined by noting the per cent of sediment in tubes filled with culture broth to a height of 13 cm. and allowed to settle in the refrigerator for four days. It can be noted from the table that only one strain, A-1, produced as much tennecetin as did the parent strain. Strain A-1 on subsequent testing did not prove to be superior to the parent strain.

When these fifteen strains from the shake flasks were subcultured on oatmeal agar, all five S strains were sporogenous, two M strains became sporogenous, three remaining mixed, and of the five A strains, two were sporogenous, two were mixed, and one remained asporogenous.

## TABLE IX

### NUMBERS AND TYPES OF COLONIES OBTAINED BY PLATING OUT PARENT STRAIN ON ASSAY OVERLAY AGAR

| Plate number | Number of Colonies | | |
|---|---|---|---|
| | S Type | A Type | M Type |
| 1 | 52 | 24 | 3 |
| 2 | 142 | 54 | 21 |
| 3 | 72 | 40 | 18 |
| 4 | 56 | 23 | 6 |
| Totals: | 322 | 141 | 48 |
| | (63.0%) | (27.6%) | (9.4%) |

TABLE X

TYPE OF GROWTH, SETTLED MYCELIAL VOLUME AND ANTIBIOTIC PRODUCTION BY DIFFERENT STRAINS OF *S. chattanoogensis*

| Strain Number | Type of Growth | SMV (per cent) | Assay (units/ml.) |
|---|---|---|---|
| parent | fine | 45.5 | 10.0 |
| S-1 | pellets | 27.0 | 3.5 |
| S-2 | pellets | 33.0 | 1.0 |
| S-3 | pellets | 19.7 | 6.4 |
| S-4 | pellets | 25.4 | 3.2 |
| S-5 | fine | 24.6 | 2.5 |
| M-1 | pellets | 33.0 | 4.0 |
| M-2 | fine | 20.8 | 2.0 |
| M-3 | pellets | 34.6 | 3.5 |
| M-4 | fine | 27.0 | 6.4 |
| M-5 | fine | 23.0 | 3.5 |
| A-1 | fine | 34.6 | 10.0 |
| A-2 | pellets | 30.8 | 2.5 |
| A-3 | pellets | 21.5 | 3.5 |
| A-4 | fine | 23.0 | 2.0 |
| A-5 | fine | 27.0 | 2.0 |

## G. Antifungal Activity of Tennecetin

Two methods were employed for determining the antibiotic spectrum of tennecetin. One was the familiar cross-streak method in which the antibiotic-producing organism was grown as a streak across the center of a plate of agar medium. After several days other organisms, against which antagonism was sought, were streaked on the same agar plate but at right angles to the antibiotic-producing organism. These "testers" were streaked right up to the line of growth of the antibiotic producer. Any inhibition of growth extending four millimeters or more from the edge of the streptomycete was taken as evidence of antibiotic activity.

In the second method, filter paper discs saturated with culture filtrates or with solutions of tennecetin were placed on the surface of agar media seeded with the test organism. Antibiotic activity was indicated, following suitable incuation, by clear zones of no growth around the paper discs.

Table XI lists the organisms found to be sensitive to tennecetin by one or both of these methods. Table XII lists organisms tested and found not to be affected by the antibiotic. It is noteworthy that no strain of yeast or mold was found to be resistant to the antibiotic activity

## TABLE XI

### ORGANISMS FOUND TO BE INHIBITED in vitro BY TENNECETIN

| |
|---|
| Absidia spinosa |
| Allescheria boydii |
| Ashbya gossypii |
| Aspergillus sp. (numerous strains) |
| Aspergillus candidus |
| Aspergillus clavatus |
| Aspergillus glaucus |
| Aspergillus fumigatus (two strains) |
| Aspergillus niger (several strains) |
| Aspergillus ochraceus |
| Blastomyces dermatitidis, mycelial phase, (several strains) |
| Blastomyces dermatitidis, yeast phase (several strains) |
| Candida sp. (numerous strains) |
| Candida albicans (numerous strains) |
| Candida krusei |
| Candida parakrusei |
| Candida stellatoidea |
| Candida tropicalis |
| Circinella tenella |
| Coccidioides immitis |
| Cunninghamella sp. |
| Cryptococcus sp. (two strains) |
| Cryptococcus neoformans (two strains) |
| Debaryomyces globosus |
| Fusarium sp. |
| Fusarium graminum |
| Geotrichum sp. (several strains) |
| Hansenula anomola |
| Hansenula mrakii |
| Hansenula silvacola |
| Histoplasma capsulatum, mycelial phase (several strains) |
| Histoplasma capsulatum, yeast phase (several strains) |
| Microsporum audouini |
| Microsporum gypseum |
| Monosporium apiospernum |
| Mucor sp. |
| Mycoderma sp. |
| Nematospora coryli |

TABLE XI

ORGANISMS FOUND TO BE INHIBITED
in vitro BY TENNECETIN
(CONTINUED)

| |
|---|
| Paecilomyces sp. |
| Penicillium sp. (numerous strains) |
| Penicillium canescens |
| Penicillium citrenum |
| Pichia membranefaciens |
| Rhodotorula sp. |
| Saccharomyces carlsbergensis (two strains) |
| Saccharomyces cerevisiae (several strains) |
| Saccharomyces fragii |
| Scopulariopsis sp. |
| Schwanniomyces sp. |
| Sporobolomyces salmonicolor |
| Sporotrichum schenckii |
| Syncephalastrum racemosum |
| Thamnidium elegans |
| Torulopsis sp. |
| Trigonopsis variabilis |
| Trichoderma sp. |
| Trichophyton mentagrophytes (several strains) |
| Trichophyton rubrum (two strains) |
| Zygosaccharomyces lactis |
| |
| Corynebacterium sp. |
| Corynebacterium diphtheriae |

## TABLE XII

### ORGANISMS FOUND NOT TO BE INHIBITED in vitro BY TENNECETIN

| |
|---|
| Alcaligenes viscosus |
| Bacillus cereus |
| Bacillus graveolus |
| Bacillus subtilis |
| Bacterium cadaveris |
| Escherichia coli |
| Micromonospora sp. |
| Mycobacterium sp. (ATCC 607) |
| Neisseria catarrhalis |
| Neisseria perflava |
| Nocardia asteroides |
| Nocardia sp. |
| Paracolobactrum sp. |
| Pseudomonas aeruginosa |
| Pseudomonas fluorescens |
| Pseudomonas fragii |
| Salmonella enteritidis |
| Salmonella typhosa |
| Serratia sp. |
| Staphylococcus albus |
| Staphylococcus aureus |
| Streptococcus pyogenes |
| Streptomyces albus |
| Streptomyces aureus |
| Streptomyces californicus |
| Streptomyces cellulosae |
| Streptomyces coelicolor |
| Streptomyces griseus |
| Streptomyces lavendulae |
| Streptomyces rimosus |
| Streptomyces viridis |

of tennecetin. On the other hand, of more than twenty species of bacteria tested, only two strains of Corynebacterium were inhibited.

In no case have resistant colonies been found in a zone of inhibition. That viable cells may still be present within these zones,however, is clear from experiments in which bits of agar from inhibition zones were subcultured to appropriate antibiotic-free media. In those instances in which this was attempted, prompt growth occurred in the subcultures. But the organisms recovered in these subcultures remained entirely sensitive to the action of tennecetin when tested again.

In an attempt to determine the concentrations of tennecetin necessary to inhibit growth of representative organisms, known amounts of the antibiotic were incorporated into Sabouraud agar, In these experiments, material obtained from Merck and Company, assaying 7.3 units of tennecetin activity per milligram, was used. The material was diluted in water to give various final concentrations in agar up to 1.0 unit per milliliter. (The tennecetin solutions were added to autoclaved, cooled media and poured aseptically into sterile Petri dishes.) The plates were inoculated with various yeasts and molds and incubated at appropriate temperatures until good growth was obtained on control media containing no antibiotic.

The results obtained from two such experiments are shown in Table XIII and Table XIV. In these tables, the figures to the right of the names of the organisms refer to extent of growth as estimated visually on a scale between 0 (no growth) and 4 (maximum growth).

TABLE XIII

CONCENTRATIONS OF TENNECETIN
REQUIRED FOR INHIBITION OF
CERTAIN PATHOGENIC FUNGI
In Vitro

| Organism | Tennecetin, u/ml. | | | |
|---|---|---|---|---|
| | 0.5 | 0.25 | 0.05 | none |
| Allescheria boydii | 0 | 0 | 4 | 4 |
| Blastomyces dermatitidis (yeast phase) | 0 | 0 | 4 | 4 |
| Blastomyces dermatitidis (mycelial phase) | 0 | 0 | 2 | 2 |
| Candida albicans | 0 | 0 | 4 | 4 |
| Candida parakrusei | 0 | 0 | 4 | 4 |
| Coccidioides immitis | 0 | 0 | 1 | 2 |
| Cryptococcus neoformans | 0 | 4 | 4 | 4 |
| Geotrichum candidum | 0 | 4 | 4 | 4 |
| Microsporum audouini | 0 | 3 | 3 | 4 |
| Sporotrichum schenckii | 0 | 4 | 4 | 4 |
| Trichophyton rubrum | 0 | 0 | 4 | 4 |

0, no growth; 4 maximum growth

TABLE XIV

CONCENTRATIONS OF TENNECETIN
REQUIRED FOR INHIBITION OF
CERTAIN NON-PATHOGENIC FUNGI
In Vitro

| Organism | Tennecetin, u/ml. | | | |
|---|---|---|---|---|
| | 1.0 | 0.1 | 0.01 | none |
| Absidia spinosa | 0 | 0 | 0 | 4 |
| Aspergillus fumigatus | 0 | 2 | 4 | 4 |
| Aspergillus glaucus | 0 | 0 | 4 | 4 |
| Aspergillus niger | 0 | 0 | 4 | 4 |
| Candida sp. | 0 | 4 | 4 | 4 |
| Debaryomyces globosus | 0 | 4 | 4 | 4 |
| Paecilomyces sp. | 0 | 2 | 4 | 4 |
| Saccharomyces carlsbergensis | 0 | 2 | 4 | 4 |
| Saccharomyces cerevisiae | 0 | 1 | 4 | 4 |
| Syncephalastrum racemosus | 0 | 0 | 4 | 4 |
| Thamnidium elegans | 0 | 0 | 4 | 4 |
| Trichoderma sp. | 0 | 0 | 4 | 4 |

0, no growth; 4, maximum growth

## H. Some Chemical Properties of Tennecetin

### Solubility

Different lots of dried powders recovered from tennecetin shake flask experiments and from larger fermentations have varied somewhat in their solubility characteristics. All have been soluble to some extent in water, methanol, ethanol, butanol, acetone, formamide, pyridine, and propylene glycol.

Solubility in water was increased when the water was made slightly alkaline. Tennecetin was found to be virtually insoluble in dilute acids. No solubility in non-polar solvents such as ether, benzene, and carbon tetrachloride was observed with any preparations.

Because of the impurities present in these solid preparations it was not possible to determine solubility in terms of weight of antibiotic per unit volume of solvent. An estimation of the relative solubility of tennecetin in various solvents was obtained, however, in the following experiment.

Using one of the lots of solid material supplied by Merck and Company, 100 milligram portions of the material were weighed out into separate flasks and ten milliliters of the solvent under test was added to the 100 mg. portion. The resulting solution or suspension

was centrifuged. When methanol, methanol plus 1 per cent calcium chloride, pyridine, formamide, propylene, glycol, and o.1N sodium hydroxide were used as solvents, all the material appeared to go into solution. Following centrifugation, there was no sediment with any of these solvents except methanol and methanol plus calcium chloride. The amount of sediment with these and other solvents was noted and then the sediment was discarded. The supernatant fluid was diluted 1:10, 1:20, and 1:40 with distilled water, and these dilutions were assayed by the standard assay method. As is shown in Table XV, considerable difference occurred in the amounts of activity in solution. On the basis of this and similar experiments, it was decided that methanol, propylene glycol, or dilute alkali were the solvents of choice. The unit definition of tennecetin, however, based on activity in water, was retained.

Ultra-Violet Absorption Spectrum

Figure 12 shows a typical ultra-violet absorption determination of a dilute methanol solution of tennecetin. The slight shoulder at or near 274 millimicrons was observed in most, but not all, determinations. Definite peaks, characteristic of tetraenes, appeared at 288, 300 - 302, and 315 - 318 millimicrons. The latter peak was always sharp, but varied between 315 and 318 in

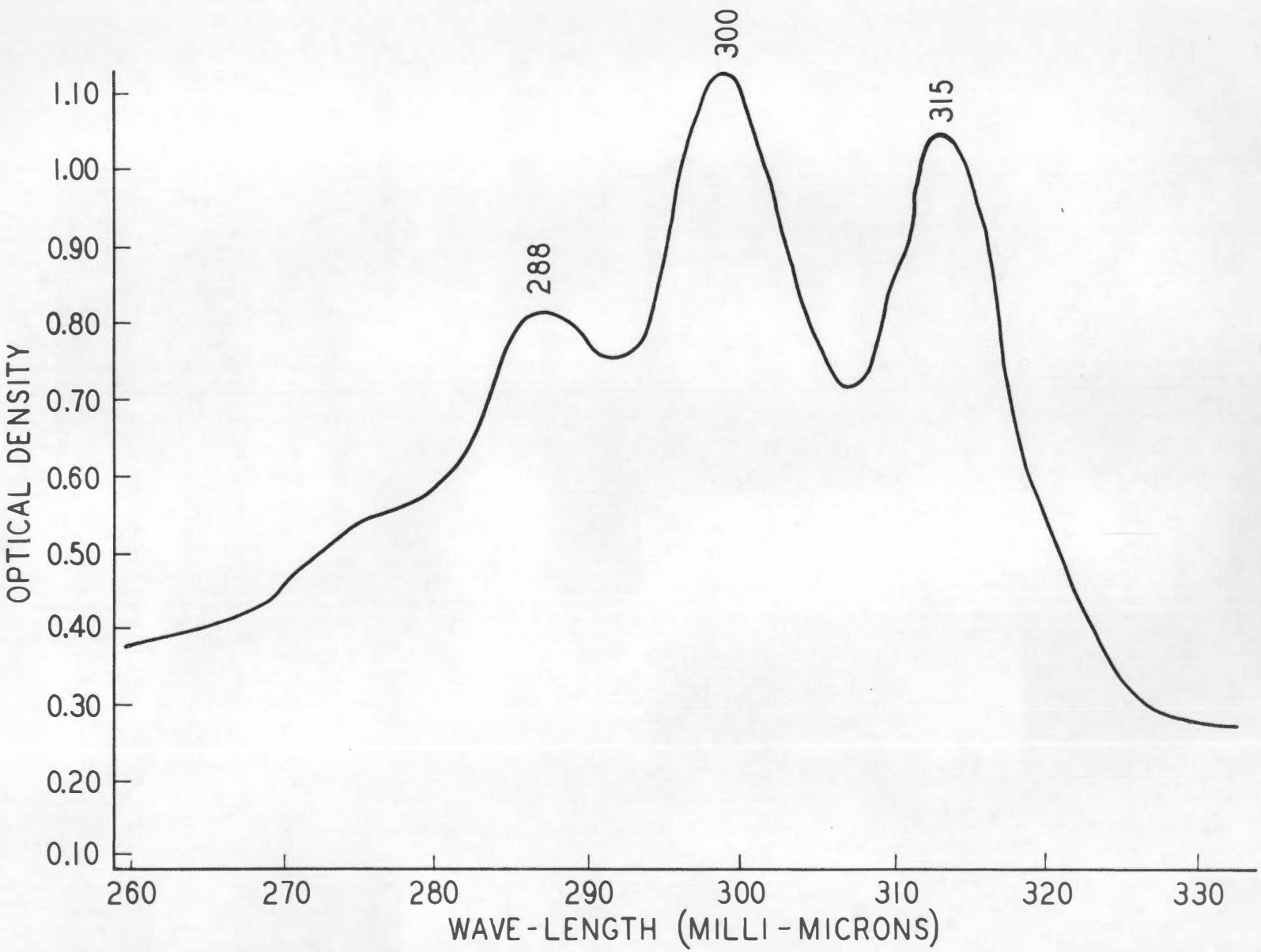





FIGURE 12. ULTRA-VIOLET ABSORPTION SPECTRUM OF TENNECETIN

TABLE XV

ACTIVITY OF TENNECETIN
IN VARIOUS SOLVENTS

| Solvent | Activity (units/mg.) |
|---|---|
| distilled water | 3.2 |
| 0.1 N sodium hydroxide | 9.5 |
| methanol | 12.8 |
| ethanol | 8.0 |
| methanol plus 1% $CaCl_2$ | 12.8 |
| propylene glycol | 10.0 |
| formamide | 10.0 |
| pyridine | 8.0 |
| acetone | 1.2 |

different determinations. No appreciable absorption was observed at wave lengths greater than 330 millimicrons.

## Infra-Red Absorption Spectrum

The infra-red absorption spectrum obtained from pressed potassium bromide pellets containing tennecetin is shown in Figure 13. Comparison of this with published spectra for other tetraene antibiotics showed several differences in the major and minor absorption bands.

Tennecetin has major bands at or near 3.0, 3.4, 6.0, 6.3, 9.5, 9.9, and 11.9 microns and bands of lesser intensity at or near 6.6, 6.9, 7.2, 7.9, 8.5, 9.05, and 11.3 microns. Minor peaks or shoulders occur at 5.85, 7.7, and 8.9 microns.

Like other reported tetraenes, tennecetin showed strong absorption at the hydroxyl band at 3.0 microns, and at 5.87 microns, characteristic of a carbonyl function (Dutcher et al., 1955).

## Qualitative Tests

Aqueous solutions of tennecetin were detrorotatory in polarized light.

Tennecetin gave a stable wine-red color in concentrated sulfuric acid. In concentrated phosphoric acid, the color was water-green. There was no color

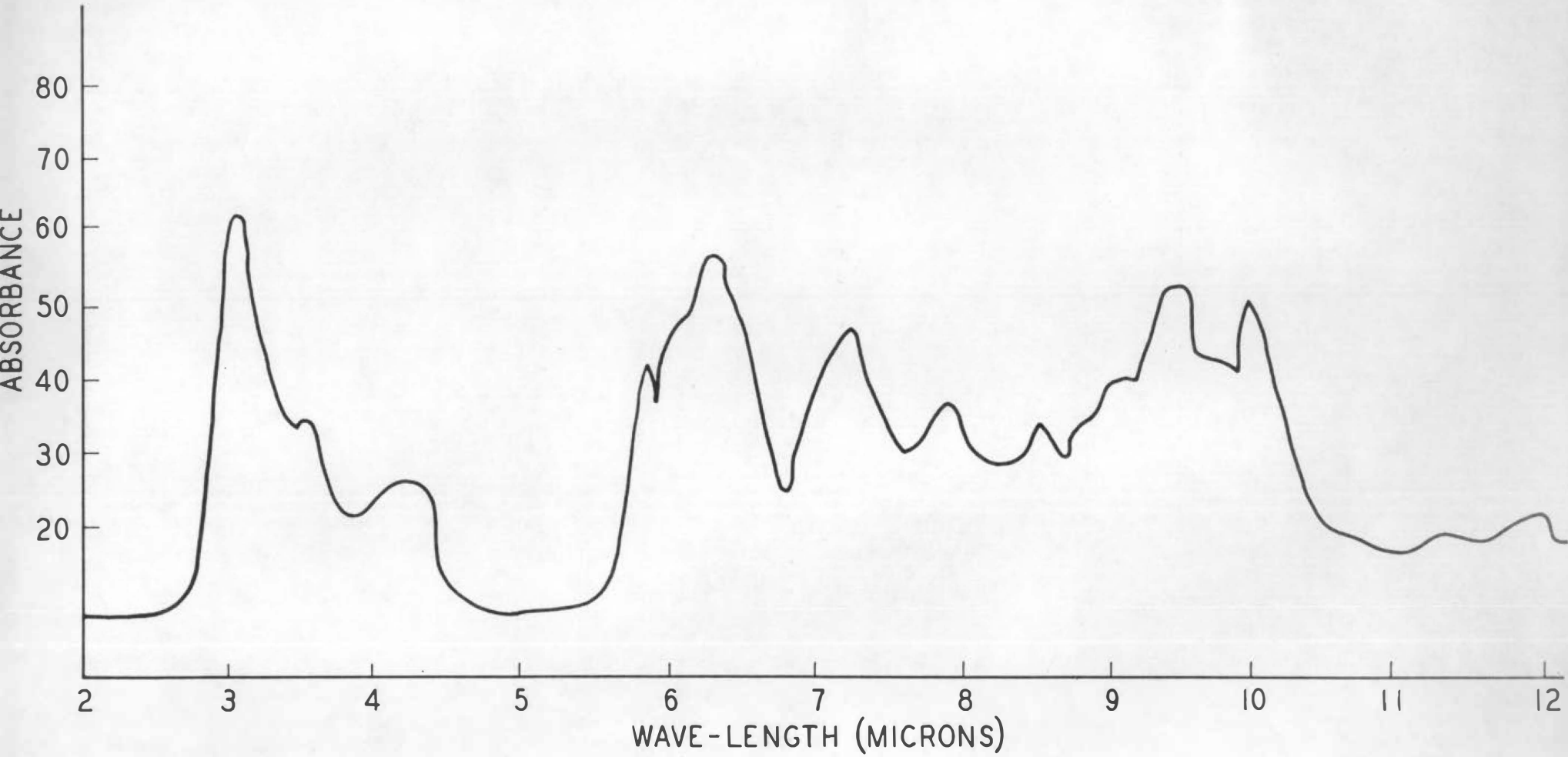



*FIGURE 13. INFRA-RED ABSORPTION SPECTRUM OF TENNECETIN*

in concentrated hydrochloric acid.

Solutions of potassium permanganate were decolorized rather rapidly by tennecetin.

The ferric chloride test for phenolic groups was negative.

The lead acetate test for sulfur was negative.

Ninhydrin and biuret tests were negative.

There was no reaction with 2-4 dinitrophenylhydrazine.

The anthrone test for carbohydrates was negative.

Fehling's test for reducing sugars was negative.

Tauber's benzidine test for pentoses was negative.

Paper Chromatography

Chromatography of tennecetin was accomplished on Whatman #1 filter paper strips, one inch wide, using the decending one-dimensional technique. Water-saturated n-butanol was used as the solvent. The procedure was carried out at room temperature, using thirty milliliters of solvent in the upper dish and 100 ml. solvent in the bottom of the jar to insure saturation of the atmosphere. Strips were chromatographed overnight and were developed by bioautography. In this procedure, the strips were first dried in air, then placed on the surface of pyrex

baking dishes containing assay overlay agar seeded with Saccharomyces carlsbergensis K-20. After fifteen minutes the strips were removed from the agar and the trays were incubated at room temperature for twenty-four hours. Zones of inhibition in areas containing antibiotic were better visualized when a solution containing 2, 3, 5, triphenyl-tetrazolium chloride and glucose was poured over the agar. After about fifteen minutes, reduction of the triphenyl-tetrazolium chloride to its red formazon by the viable yeast gave the trays a red background and sharply damarkated colorless zones of inhibition. $R_f$ values were calculated by the usual methods, using the center of the zone of inhibition as the point to which movement was measured.

In all chromatography experiments, the deep yellow color of tennecetin preparations remained at the point of application ($R_f$ 0.0). Tennecetin never gave more than a single zone, indicating that it is not a mixture of antibiotics and does not contain more than a single active component. Tennecetin in different determinations gave $R_f$ values between 0.33 and 0.51 with water-saturated n-butanol as the solvent. When the aqueous layer of this mixture was used as the solvent, the $R_f$ value was 0.86. With a solvent composed of equal parts distilled water and acetone, the $R_f$ value for tennecetin was 0.74.

When mixtures of tennecetin, nystatin, and rimocidin

sulfate were chromatographed on the same strip, three distinct zones were obtained. A tracing of the results obtained in an experiment in which the three antibiotics were chromatographed at the same time on separate strips, using wet butanol as the solvent, is shown in Figure 14. The value obtained for nystatin, 0.22, is in good agreement with the $R_f$ value of 0.25 obtained by Ammann and Gottlieb (1955) with the same solvent system.

## I. Toxicity and Other Pharmacological Properties

### Rabbits

Barr (1959) has reported toxicological properties of tennecetin in rabbits and other animals. He found the $LD_{50}$ for rabbits to be, in units per kilogram, 200 by the intravenous route, 1,200 by the intramuscular route, 685 intraperitoneally, 1,800 by subcutaneous injection, and greater than 2,000 orally.

We have not determined $LD_{50}$ values in rabbits, but have used this species to study absorption and blood concentrations of tennecetin. In most of these experiments, no activity could be detected in serum or plasma by the standard plate assay method. However, when the plate assay was modified by reducing the amount of inoculum and pouring the overlay agar very thinly, zones of activity could be detected against Saccharomyces carlsbergensis. No

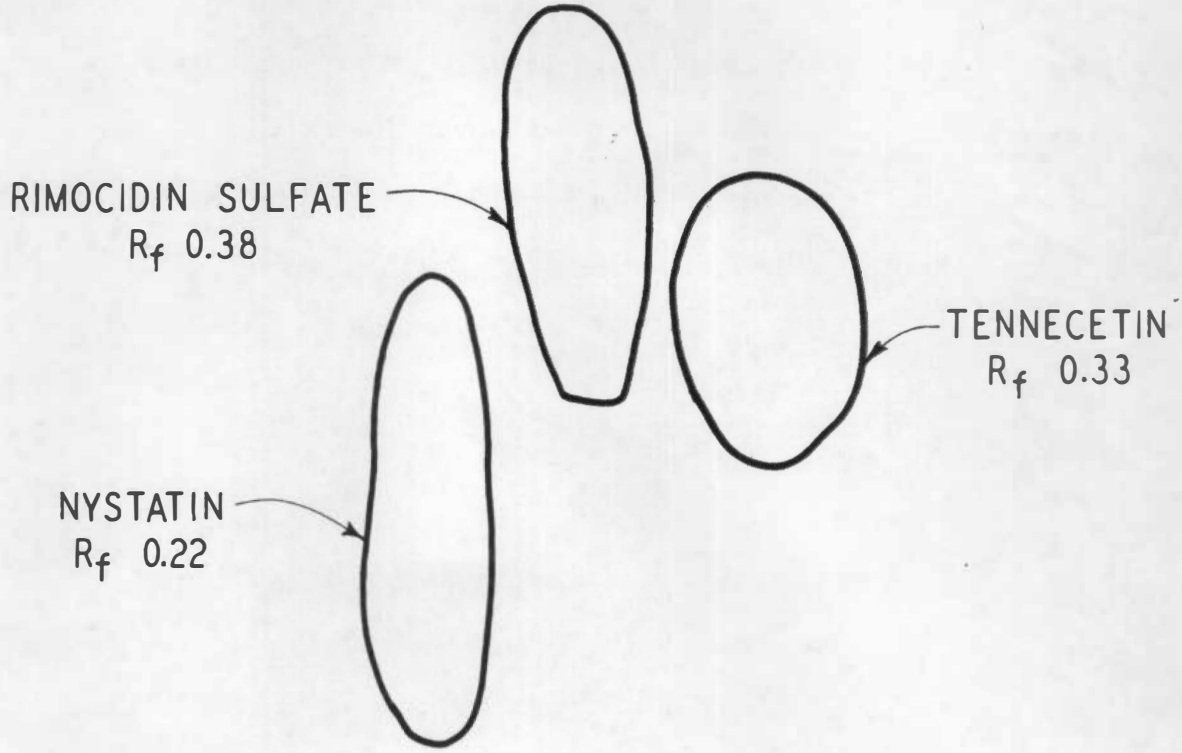



**Figure 14. Tracing of "Bioautographic Plate" following paper Chromatography of Tennecetin, Rimocidin Sulfate, and Nystatin.**

quantitative data could be obtained by this method, although estimates of values between 0.1 and 0.5 units per milliliter were made.

The following experiments illustrate our experiences with rabbits.

Experiment 1:

Five milliliters of an aqueous solution of tennecetin (Merck lot), adjusted to pH 6.8 and containing 250 milligrams of tennecetin, was injected intravenously into a rabbit weighing approximately five pounds. There was no reaction to the injection.

Blood was drawn sixty minutes after injection and assayed by the standard plate method. No activity was detected. Urine collected at twenty and sixty minutes after injection showed slight activity. The sixty minute urine sample contained about 0.5 units/ml.

This rabbit appeared in good health when observed for one week following injection.

Experiment 2:

A neutral aqueous suspension containing 200 units/ml. was injected intravenously into a rabbit. When about three milliliters of the suspension had been injected, the animal went into convulsions and died. Death appeared to be due to an embolism.

Blood drawn from the heart two minutes after death and assayed by the standard plate method revealed 2.0 units/ml. activity. Urine taken from the bladder at this time showed no activity.

Experiment 3:

A rabbit weighing about eight pounds was injected subcutaneously with twenty-five milliliters of an aqueous neutralized solution of tennecetin (Merck lot) containing one gram of tennecetin. Blood was drawn from the marginal ear vein at 2, 3, 4, 5, and 8 hours after injection. Urine was collected at 3, 4,5, and 9.5 hours. Serum and urine were assayed by the more sensitive but qualitative modification of the plate assay method.

Using this modification, all samples of serum and urine showed slight activity. In the case of serum, this

activity was estimated to be of the order of 0.1 units/ml.

This rabbit was found dead six hours later. Post mortem examination of the injected site showed no deposit of the injected material.

Experiment 4:

Experiment 3 was repeated using a 2,000 gram rabbit and three grams of tennecetin. Slight but definite activity was again found in the serum at 1, 2, 3, 6, 8, and 24 hours, and in the urine at 8.5 hours. (This was the only urine sample tested.)

The animal was sacrificed at 24 hours. Post mortem examination revealed no gross pathology of the internal organs. At the site of injection much of the injected material was found; it was present as a thick, viscous, light brown material, similar in appearance to the heavy slurry that had been injected.

No local inflammatory response was noted. Microscopically there were no animal cells in the material. Assay of the residual antibiotic content showed that much activity remained. Undiluted, the material gave zones of 35 mm. on the standard plate assay.

Experiment 5:

Twenty-two milliliters of a propylene glycol solution of tennecetin was injected into a rabbit weighing about five pounds. The solution contained 40 mg./ml. of Merck-produced tennecetin. The injection was made into the muscles of the abdomen.

The rabbit was bled at 1.5, 3, and 5 hours after injection. Using the more sensitive modified plate assay method, antibiotic activity was detected in the 1.5, 3, and 5 hour samples, but not in a sample taken at 6.5 hours. Urine showed antibiotic activity at 3 hours, but not at 6.5 hours.

Experiment 6:

A five pound rabbit was infused intravenously with 40 ml. aqueous solution of tennecetin, pH 9.5, containing 28 units/ml. Thirty-five milliliters of the infusion was administered within 30 minutes. The rabbit exhibited some signs of distress during the infusion, but no frank reaction.

Blood was drawn by cardiac puncture three hours after starting the infusion. The rabbit died five hours

later. Blood serum and sodium hydroxide-hydrolyzed whole blood taken at three hours and also post mortem showed slight antibiotic activity by the modified plate assay method. Because of the slight amount of activity recovered, it was not possible to determine if the hemolyzed whole blood gave definitely higher assay values than serum.

Experiment 7:

A rabbit weighing six pounds was injected intravenously with an aqueous solution (pH 9.5) containing 50 mg. tennecetin per milliliter. When approximately 3.5 ml. had been administered, the rabbit suddenly began gasping for breath, and died within two minutes. Heart's blood drawn within five minutes after death assayed 0.7 units/ml.

Another rabbit receiving 2.0 ml. intravenously of the same solution (50 mg./ml.) showed immediate signs of acute distress, but appeared to have recovered completely within thirty minutes. One hour after the first injection, another intravenous injection of the same solution was attempted. When about 1.5 ml. had been introduced, the animal went into a severe reaction and died. Autopsy revealed a red, engorged liver and hemorrhagic areas on the inner muscular wall of the abdomen.

Mice

Two experiments were performed to determine the acute toxicity of tennecetin in mice. Material for both of these experiments was dried solids obtained from shake flask experiments. The mice were CFW males weighing 18 - 20 grams each. Aqueous solutions of tennecetin were injected intraperitoneally, 0.4 ml. per mouse. In the first experiment, material assaying 217.5 units per mg. was used. In the second experiment, material assaying 100 units per mg. was used.

Table XVI and Table XVII show the results of these two experiments.

Our results indicate higher $LD_{50}$ values in mice than those reported by Barr (1959, loc cit.). He reported the $LD_{50}$ in mice injected intraperitoneally as 1,250 units/Kg. Our value, taken from Table XV is 8,156 units/kg., and from Table XVI 13,400 units/Kg.

As the material used in the experiment reported in Table XVI assayed only about one-half as high as that used in the experiment reported in Table XV, it would appear that toxicity is increased with increasing purity. However, this is not in agreement with the data of Barr, who used material of considerably lower purity (approx. 10 units/mg.)

Two experiments were performed to determine the chronic (oral) toxicity in mice. In the first of these experiments, ten young CFW mice were given onlypowdered rat chow. Ten similar mice were given the same powdered food, but with tennecetin added at a level of 800 mg. tennecetin per 150 grams of chow. The animals received water ad lib., but received no food except the weighed amounts presented at feeding times.

At the end of twelve days, all test and control animals were living and well. The test animals had received a total of 1,200 units of tennecetin per mouse (100 units/day/mouse). Test animals gained weight during the experiment at the same rate as did control animals.

TABLE XVI

ACUTE TOXICITY OF CRUDE TENNECETIN IN MICE (ASSAY: 217.5 u/mg.)

| Mouse Number | Dose mg./Kg. | Dose units/Kg. | Result |
|---|---|---|---|
| 1 | 150 | 32,625 | dead at 4 hours |
| 2 | 150 | 32,625 | dead at 4 hours |
| 3 | 150 | 32,625 | dead at 4 hours |
| 4 | 150 | 32,625 | survived |
| 5 | 75 | 16,313 | dead at 5 hours |
| 6 | 75 | 16,313 | dead at 18 hours |
| 7 | 75 | 16,313 | dead at 18 hours |
| 8 | 75 | 16,313 | dead at 18 hours |
| 9 | 37.5 | 8,156 | dead at 24 hours |
| 10 | 37.5 | 8,156 | survived |
| 11 | 37.5 | 8,156 | survived |
| 12 | 37.5 | 8,156 | survived |
| 13 | saline | none | survived |
| 14 | saline | none | survived |
| 15 | saline | none | survived |
| 16 | saline | none | survived |

TABLE XVII

ACUTE TOXICITY OF CRUDE TENNECETIN IN MICE (ASSAY: 100 u/mg.)

| Mouse Number | Dose mg./Kg. | Dose units/Kg. | Result |
|---|---|---|---|
| 1 | 134 | 13,400 | dead at 24 hours |
| 2 | 134 | 13,400 | dead at 26 hours |
| 3 | 134 | 13,400 | survived |
| 4 | 134 | 13,400 | survived |
| 5 | 90 | 9,000 | survived |
| 6 | 90 | 9,000 | survived |
| 7 | 90 | 9,000 | survived |
| 8 | 90 | 9,000 | survived |
| 9 | 53.6 | 5,360 | survived |
| 10 | 53.6 | 5,360 | survived |
| 11 | 53.6 | 5,360 | survived |
| 12 | 53.6 | 5,360 | survived |
| 13 | 26.8 | 2,680 | survived |
| 14 | 26.8 | 2,680 | survived |
| 15 | 26.8 | 2,680 | survived |
| 16 | 26.8 | 2,680 | survived |

Several assays of feces of test mice during the experiment showed distinct antibiotic activity. No such activity could be observed in urine, indicating poor absorption from the intestinal tract.

At the end of the twelve day feeding period, one mouse was sacrificed. Organs were excised and ground in powdered glass and methanol. After standing thirty minutes, the pooled organ emulsion (lungs, heart, liver, kidney) was centrifuged and the supernatant assayed. No activity was observed on plates eighteen hours later.

The intestines were ground similarly and assayed. Good activity was observed -- about 1 unit/ml. supernatant -- considerably more than the amount of activity found in feces alone.

In a second experiment, the same methods were used, but the level of tennecetin in the food was increased. In this experiment, each mouse received 300 mg. tennecetin in each day's food for seven days. No toxicity was observed at the end of seven days or at any time later.

Other Animals

Four hamsters weighing 60 grams each were injected intraperitoneally with a solution containing 10 mg. (73 units) of tennecetin (166 mg./Kg.). Eighteen hours later three of these animals were found dead. The fourth

hamster survived and remained well.

Two rats were injected intraperitoneally with 2.0 ml. of an aqueous solution containing 20 mg./ml. tennecetin. One of these animals, weighing 250 grams, was sacrificed two hours later. No antibiotic activity could be found in extracts of the internal organs. The other animal, weighing 470 grams, was found dead eight hours after injection.

Baby chicks, kept from age one day to nineteen days on a diet including large amounts of spent broth solids from a Merck tennecetin fermentation thrived and showed no signs of toxicity. The spent broth solids contained demonstrable tennecetin activity, but less than 1 unit/mg. Eight chicks on a regular diet increased in weight from 300 to 1,044 grams (37.5 to 130.5 grams per chick average) during the 19-day period. Eight chicks receiving the same food but with 0.5 grams spent broth solids per pound of food increased in weight from 300 to 1,126 grams (37.5 to 140.7 grams per chick average).

Activity in Presence of Blood Plasma and Serum

To 1.8 ml. fresh human serum was added 0.2 ml. of a tennecetin solution containing approximately 60 units/ml. Similarly, 0.2 ml. of the same solution was added to 1.8 ml. normal saline.

These mixtures were allowed to stand at 37°C for two hours, after which they were diluted with water and assayed by the standard plate assay.

The saline-antibiotic control assayed 60 units/ml.

The serum-antibiotic test mixture assayed 42 units/ml.

The results of a similar experiment, in which fresh human plasma was used as the diluent, are shown in Table XVIII. In this experiment, the mixtures were incubated for three hours before being assayed.

## J. Stability Studies

Solutions of tennecetin kept at refrigerator temperature have maintained full activity for three weeks or more.

Tennecetin is more stable in acid than in alkaline solutions. This is shown in Table XIX, which gives comparative assay volues for the same amount of tennecetin held for ninety minutes in solutions containing 0.05 N hydrochloric acid and 0.05 N sodium hydroxide.

Table XX shows the results obtained when portions of a 72-hour shake flask culture were adjusted to pH 4.0, pH 7.0, and pH 10.0, and treated as follows: a) not heated; b) placed in a boiling water bath 5 minutes; c) placed in a boiling water bath for 10 minutes;

TABLE XVIII

ACTIVITY OF TENNECETIN SOLUTIONS
DILUTED IN FRESH HUMAN PLASMA

| Sample Number | Dilution | Diluent | Activity (units/ml.) |
|---|---|---|---|
| SF102 | 1:10 | normal saline | 23.0 |
| SF102 | 1:10 | plasma | 12.0 |
| SF102 | 1:4 | normal saline | 18.4 |
| SF102 | 1:4 | plasma | 8.0 |
| 5031 | 1:2 | normal saline | 5.8 |
| 5031 | 1:2 | plasma | 3.3 |
| 5031 | 1:3 | normal saline | 6.0 |
| 5031 | 1:3 | plasma | 3.9 |

d) placed in a boiling water bath 20 minutes; e) autoclaved at 121°C for 30 minutes.

An experiment was designed to determine the effect of visible light on tennecetin. In this experiment, portions of a solution of tennecetin dried powder were placed in ordinary glass test tubes. Two of the tubes were wrapped in tinfoil and served as controls. One tube was exposed to the light of a window for twenty hours. Another was placed 15 cm. from a burning 60 watt electric light bulb for twenty hours. A fifth tube was placed in a dark place in the refrigerator as a control.

Table XXI shows that there was only slight, if any, inactivation of tennecetin by visible light. (The amount of inactivation noted is within the limits of assay error.)

In experiments designed to determine the effect of ultra-violet light on tennecetin, the following procedure was used. Solutions of the antibiotic were placed in Petri dishes with lids removed (20 ml. per dish) and exposed for varying intervals of time to the light of a General Electric 15 watt germicidal lamp. The distance from the lamp to the solution was four inches. At intervals, one milliliter portions of the solution were withdrawn for assay. In other experiments the distance from the lamp was varied between 3.5 and 16.0 inches, and control solutions containing a reducing agent were included.

TABLE XIX

STABILITY OF CRUDE TENNECETIN
IN ACID AND IN ALKALI

| Treatment | Assay (units/ml.) |
|---|---|
| none | 4.0 |
| 0.05 N HCl | 2.4 |
| 0.05 N NaOH | 0.0 |

TABLE XX

HEAT STABILITY OF TENNECETIN SOLUTIONS
AT pH 4.0, pH 7.0, AND pH 10.0

| Treatment | Assay (units/ml.) | | |
|---|---|---|---|
| | pH 7.0 | pH 4.0 | pH 10.0 |
| none (control) | 8.0 | 8.0 | 8.0 |
| boiled, 5 minutes | 8.0 | 8.0 | 5.2 |
| boiled, 10 minutes | 8.0 | 6.6 | 2.7 |
| boiled, 20 minutes | 8.0 | 3.3 | 0.8 |
| autoclaved, 15 minutes | 3.3 | 0.0 | 0.0 |

TABLE XXI

STABILITY OF TENNECETIN SOLUTIONS TOWARD VISIBLE LIGHT

| Treatment | Assay (units/ml.) |
|---|---|
| At window, 20 hours | 6.2 |
| At window, wrapped, 20 hours | 6.0 |
| Under electric light bulb, 20 hours | 5.0 |
| Under light bulb, wrapped, 20 hours | 5.6 |
| Refrigerated in dark, 20 hours | 6.6 |

Figure 15 shows the results obtained in one such experiment in which the solution originally contained 22.5 units/ml. and the distance between lamp and solution was 3.5 inches. In this and in similar experiments, addition of ascrobic acid (5 mg./ml.) completely prevented destruction of antibiotic activity by ultra-violet light.

## K. Miscellaneous Observations

Tennecetin was not adsorbed on Seitz filter pads.

Tennecetin could be adsorbed from cultures and from solutions with activated charcoal. However, all attempts to elute the activity from the charcoal were unsuccessful.

When acetone extracts of cultures on agar media were allowed to stand several days in the refrigerator, a yellow sediment settled out. When this sediment was recovered by centrifugation, washed in ether and dried, a deep yellow amorphous solid was obtained. This material was completely insoluble in all solvents tested. Bits of the insoluble material placed on assay plates showed no antibiotic activity.

Tennecetin appeared to be more active in an alkaline than in an acid medium. In an experiment, the results of which are shown in Table XXII, the same solution was assayed on plates containing overlay agar adjusted to

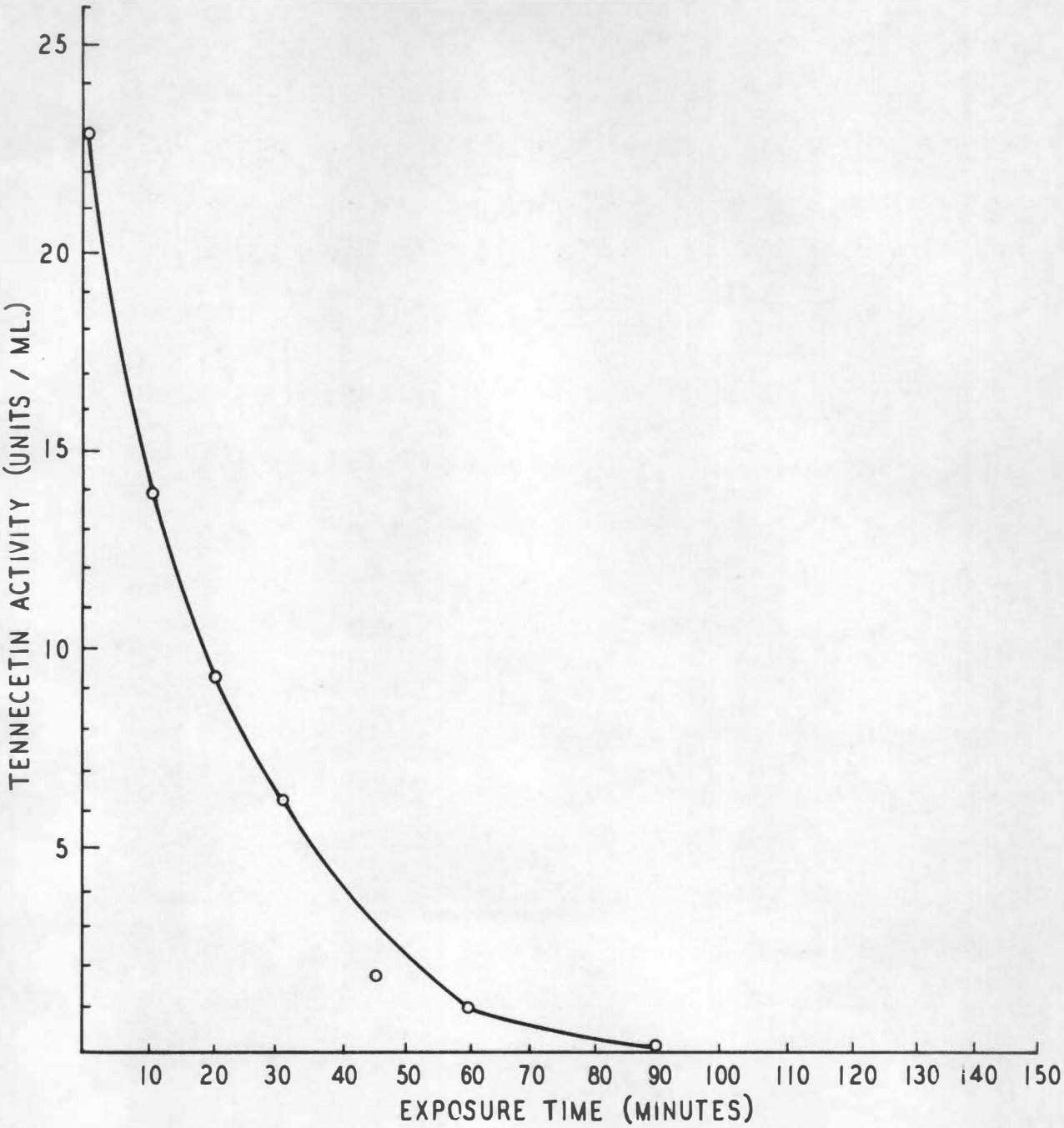



Figure 15. Destruction of Tennecetin Activity by Ultra-Violet Light.

pH 5.0, pH 7.0, and pH 9.0. As can be seen from the table, almost twice as much activity, as measured in units, was present as the pH of the medium increased from pH 5.0 to pH 9.0.

TABLE XXII

EFFECT OF pH OF MEDIUM ON ACTIVITY
OF TENNECETIN AGAINST
*Saccharomyces carlsbergensis*

| pH | Assay (units/ml.) |
|---|---|
| 5.0 | 5.2 |
| 7.0 | 6.4 |
| 9.0 | 10.0 |

# CHAPTER IV

## DISCUSSION

> The surest way to be a bore is to leave nothing unsaid. (Attributed to Voltaire.)

An organism which appears to be a new species of the genus Streptomyces has been isolated from soil and studied in the laboratory. The organism, for which the name Streptomyces chattanoogensis is proposed, is able to produce a tetraene antibiotic not previously described. The organism is of interest because of its ability to produce this antibiotic and also as a new species in a well-known genus. The antibiotic, which we have named tennecetin, is of interest because of its possible usefulness as an antifungal agent in medicine and agriculture, and also as an example of a unique class of compounds (polyenes) produced by streptomycetes.

In discussing these findings, it is necessary to re-state the original purpose of this investigation. That purpose, as outlined very early in our work, has been to study a previously unknown antibiotic from all aspects possible within the limits of our time and resources, with a view toward establishing the basic information necessary for further possible work, and, at the same time

to learn the initial or exploratory phases of antibiotic research from an actual experience with a specific new antibiotic. Adherence to these purposes has at times made it necessary to stop short of completion of problems that appeared to cry out for solution.

It is recognized, for example, that tennecetin, as herein described, is an incompletely characterized antibiotic. No claim for therapeutic efficacy can be made on the basis of the data presented. The chemical information so far obtained identifies the antibiotic only as a tetraene with certain properties apparently common to that class of compounds. Tennecetin has not been obtained in crystalline form. Much other desireable information is lacking, and many questions of practical and theoretical importance must be answered before the value and significance of this antibiotic can be assessed.

Indeed, our experience has been that one of the chief frustrations in antibiotic research is the individual worker's inability to encompass the whole of his field. Should he stake out a small area for his investigation -- say the mode of action of a given antibiotic, or strain variation in a single streptomycete -- he might expect the satisfaction of answering a few specific questions. But if he wishes to survey all the possibilities of a new antibiotic, he is continually distracted by

new problems that arise at a considerably faster rate than that at which old problems can be solved. The price for such an approach thus appears to be a superficiality that is not intellectually satisfying. On the other hand, such a ground-breaking chore seems to be essential for each new antibiotic, and constitutes the basis upon which later studies may be built. The present investigation, therefore, is an example of this kind of preliminary surveying. That it has not been entirely in vain is indicated by the fact that problems and possibilities it has uncovered have become subjects of research for other workers.

It was stated earlier that some speculations preceded this work. It may be pertinent at this point to give an account of some of these speculations.

Perhaps the only prejudice with which this study was begun was the idea that antibiotics are not discovered, but are developed. Although this assumption on the surface may not appear to carry any great implications, it is at direct variance with a philosophy that has dominated commercial antibiotic research programs for at least fifteen years. According to this philosophy, new antibiotics begin with a so-called "screening program" in which literally thousands of soil samples -- collected mostly from far-away places -- are surveyed for their

content of organisms capable of producing antibiotics when tested by a standard screening test. The idea behind these programs is that there exist in nature countless antibiotic-producing organisms awaiting to be found. If, then, one surveys enough soil samples, one will sooner or later encounter the desired antibiotic. The situation is much like prospecting for gold; indeed, there are obvious analogies between gold-seeking and the search for new antibiotics.

This approach to antibiotic discovery was probably strengthened by the early success of streptomycin, a graduate of a screening program that flunked some 10,000 streptomycetes (Waksman, 1948). On the other hand, a still earlier and at least equally successful antibiotic, penicillin, probably would not be discovered in most of the screening programs in use today. This is because the original mold that produced penicillin in Fleming's laboratory did so only in small quantity. By today's standards, that mold would be considered unpromising as a source of a useful antibiotic. Similarly, the various difficulties met with in penicillin production -- low yields, instability, difficulties in purification -- again if met with today would probably be sufficient to discourage further work on the mold and its antibiotic. It would be much more attractive to return to the screening program in

search of an organism that produced an apparently better antibiotic from the very beginning.

That mass screening surveys have not lived up to their theoretical promise seems evident enough. Jawetz (1956) has remarked:

> One marvels at the extraordinary stroke of fortune that brought to light penicillin, still the most outstanding agent in its field. The enormous antibiotic screening program certainly has not yielded the variety and quality of drugs that might be anticipated from the scope of the effort.

The difficulty appears to be two-fold. First, the supply of useful antibiotics in nature is probably limited. The limits might be quite high, and many new compounds may remain to be discovered, but there is none-the-less a definite limit. Support for this contention is found in the fact that a striking number of recently-discovered antibiotics have been discovered simultaneously in several laboratories (tetracycline, novobiocin, etc.). Also, it is now evident that most of the known and useful antibiotics can be grouped into a rather small number of chemical classes -- tetracyclines, polypeptides, polyenes, etc. -- individual members of the same class differing only slightly from each other, sometimes in no more than one functional group. It has been the experience of many screening programs that once a new antibiotic is discovered and studied, it appears and is recognized in many other

instances in various screening programs. This would indicate that the antibiotic has been seen many times before, but the screening program missed it. In a stimulating review of problems in the search for microorganisms producing antibiotics, Routien and Finlay (1952) make the interesting statement:

> It is already apparent that with each discovery of a new and clinically valuable antibiotic it becomes more difficult to find another.

This again would indicate that the supply of good antibiotic-producing organisms in nature is limited.

A second difficulty of the usual screening program is that it expects its organisms to perform well too soon. It seems entirely possible that an organism that produces no antibiotic on one medium, a barely recognizable amount on a second, and much on a third, will be discarded if only the first two media are used in the screening program. Furthermore, a given strain of an organism may have a very high inherent antibiotic-producing potential. Genetic manipulation may bring out this potential. When the proper conditions for antibiotic production are found, and when the highest possible antibiotic-producing mutant strains are developed, the culture may be much more attractive than some of its rivals which performed well

on the primary screening test. Kelner (1949) found that by ultra-violet and X-ray irradiation he could recover antibiotic-producing mutants from non-producing parent strains.

This study of tennecetin did not follow a screening program. Streptomyces chattanoogensis was isolated from the very first soil sample tested. From that same soil sample another streptomycete, which produced an anti-bacterial antibiotic, was also recovered. Neither organism appeared to have marked activity on primary isolation. Streptomyces chattanoogensis very shortly, however, captured our interest because of its activity against yeasts and molds.

It must be admitted that attempts to improve the antibiotic-producing capacity of the organism have not been successful. All attempts to produce mutants by ultra-violet irradiation and by natural selection failed to produce a single strain with higher capacity than the parent strain. Some improvement was made, however, by media selection, as has been shown. Further improvement of the strain seems possible, but has not so far been accomplished. If our original thesis is correct, almost any streptomycete, by proper manipulation, can be induced to produce increased amounts of a given metabolite. The history of industrial fermentations would seem to support

this thesis, for there is a large body of experience and literature on strain improvement, not only with streptomycetes, but with bacteria, yeasts, and molds as well. The most conspicuous examples of this type of improvement in yields of antibiotics by strain selection are those of penicillin (reviewed by Raper and Alexander, 1945) and streptomycin (Savage, 1949). Unfortunately, proof of this contention in the case of tennecetin would require an investment of time and effort beyond our means.

Therefore, this work does not establish our original premise, namely, that antibiotics are not "discovered," but are developed. Furthermore, the fact that the organism studied here happened to have been isolated from the first soil sample plated out does not decide the question of whether or not this was a fortunate stroke of chance or whether similar results might have been obtained starting from most any other sample of soil.

No apology is offered for describing the streptomycete studied here as a new species. In an experience covering work with several hundred strains of streptomycetes, we have never seen another culture quite like it. On the other hand, a still more extensive experience might indicate that Streptomyces chattanoogensis is merely a variety of a species already described. The name Streptomyces chattanoogensis was therefore adopted as being a

suitable and convenient designation for a strain to which we would have constant reference. It is recognized that a subsequent taxonomic study may invalidate this name.

The medium described here as GPY medium was found to support excellent synthesis of tennecetin. Economic considerations were not entertained in the development of this medium. Most of its ingredients are too costly for large-scale commercial production. But this was one phase of antibiotic production with which we did not concern ourselves. It would seem that once it can be demonstrated that a desireable antibiotic can be produced in good yield by laboratory methods, modern technology can then meet the demands of economics.

Although no attempt has been made here to show a usefulness for tennecetin as an antifungal antibiotic, certain of its properties indicate that it may have practical uses in agriculture and medicine. Amond these properties are its broad range of activity against yeasts and molds, its solubility and stability characteristics, and its relatively low toxicity.

# CHAPTER V

## SUMMARY

> Truth, Sir, is a great coquette; she will not be sought with too much passion, but often is most amenable to indifference. She escapes when apparently caught, but gives herself up if patiently waited for; revealing herself after farewells have been said, but inexorable when loved with too much fervor.
>
> E. Renan, in his speech welcoming L. Pasteur into the French Academy, 1882.

An organism isolated from soil in Chattanooga, Tennessee, was found to produce an antibiotic active against fungi. The organism -- a streptomycete -- could not be identified as a previously described species. It has been named Streptomyces chattanoogensis. Certain of its cultural characteristics have been studied and described.

The antibiotic produced by Streptomyces chattanoogensis was found to be a tetraene, not identical with any previously described tetraene. It has been named "tennecetin".

Methods were developed for the production of tennecetin in stationary and agitated cultures. A medium containing peptone, phytone, beef extract, yeast extract, and glycerol was found to give good

yields of the antibiotic. Inositol, dextrin, and galactose were found to be satisfactory replacements for glycerol as the carbohydrate source in this medium.

A microbiological assay medium for tennecetin was developed and described. A unit of tennecetin activity was arbitrarily defined.

Recovery of the antibiotic from liquid cultures was achieved by extraction with n-butanol, concentration by evaporation of the butanol, and precipitation with ether. The resulting crude solid preparations were yellow in color and soluble in water, lower alcohols, pyridine, and formamide. They were insoluble in non-polar solvents such as ether, benzene, and carbon tetrachloride. Certain other chemical and physical characteristics of tennecetin have been studied, including its ultraviolet and infra-red absorption spectra.

Tennecetin was found to have a wide range of activity against yeasts and molds. No fungus natively resistant to the action of the antibiotic was found. Tennecetin was found to have virtually no activity against bacteria.

Pharmacological studies were performed in rabbits, mice, and other animals. The acute $LD_{50}$ of tennecetin for mice injected intraperitoneally

was approximately 8,000 - 12,000 units per kilogram. Toxic doses in mice were not reached orally. Tennecetin did not appear to be readily absorbed from the intestine.

Stability studies were performed and reported.

Attempts to improve tennecetin yields by strain selection were unsuccessful.

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