THE EFFECT OF TRYPsin ON THE PATHOGENESIS OF TUBERCULOSIS

DISSERTATION

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WARREN E. ENGELHARD, B.Sc., M.Sc.

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Approved by:
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>16</td>
</tr>
<tr>
<td>EXPERIMENTAL RESULTS</td>
<td>35</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>59</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSIONS</td>
<td>71</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>73</td>
</tr>
<tr>
<td>AUTOBIOGRAPHY</td>
<td>77</td>
</tr>
</tbody>
</table>
INTRODUCTION

The utilization of the antibiotics and chemotherapeutic agents, particularly streptomycin, hydrazide of isonicotinic acid and para amino salicylic acid have, in the majority of the cases, improved the therapy of tuberculosis.

However, present therapeutic measures fail to cure more than half of the cases of tuberculous empyema, due to the fact, that they fail to attack directly, or remove the abscess membrane. Therefore, these agents are not the final answer for the complete recovery and cure from this disease. Thus, in addition to agents to suppress growth of the tubercle organism, an agent is also required which will expose the organism from the protection of its avascular tubercle. Such a process would enable the chemotherapeutic agents as well as the specific antibodies and phagocytic cells of the body to contact the bacilli and enhance their destruction.

In the search for such an agent, recent investigations indicate the value of the enzyme trypsin as an adjunct to the beneficial effects of chemotherapeutic agents in tuberculous empyema. Its proteolytic action hydrolyzes denatured protein (28) removing the protective tubercle with its coating of fibrin (33), while at the same time having no injurious effects on the functions of normal cells (32). Results
of trypsin therapy in other disease conditions emphasize an increase in the number of circulating phagocytic cells (11) and the destruction of extracellular bacterial enzymes and toxins (35). Furthermore, it has been shown that trypsin will accelerate the disappearance of an inflammatory process (21).

Up to the present time, its use has been mainly for debridement in conditions where necrotic tissues are present (32), as an adjunct to general surgical treatment of amputation stumps, osteomyelitis, decubitus ulcers (26), infected compound fractures, subcutaneous hematomas, second and third degree burns (39), gangrene, soft tissue abscesses, sinusitis, fistulas, and gun shot wounds (10). Intrapleural application on the other hand, has been restricted for the most part to tuberculous empyema, or empyema caused by mixed bacterial flora and post-operative and post-traumatic hemotorax (9, 22, 34).

The superiority of the debridement action of trypsin both in general surgery and in tuberculous empyema is usually associated with its distinctive proteolytic action. However, among the other effects produced by this enzyme cited above, may be added the rapid disappearance of tubercle bacilli from infected pleural tissue and fluids after intrapleural lavage (34), an action which has not yet been adequately explained. It was mainly for this reason that the present experiments were designed to attempt to measure the
effects of trypsin on the various properties of *Mycobacterium tuberculosis*, such as its growth, morphology, surface alterations, staining reactions, resistance to phagocytosis, and pathogenicity. In addition, the antibacterial action of the enzyme both *in vitro* and *in vivo* was determined and an attempt was made to correlate these effects with the successful clinical actions of trypsin.
LITERATURE REVIEW

Trypsin exerts certain actions which are fundamental in combating infectious processes (11,21,32,34), particularly diseases with the pathology exhibited by a tuberculous process. Although these characteristics are not well understood, trypsin is well known as one of the main enzymes of the animal digestive tract and has been extensively investigated.

The most common source of trypsin is animal pancreas. In this gland trypsin occurs as the precursor trypsinogen, together with other precursors such as chymotrypsinogen. This discussion and research is limited to that trypsic enzyme occurring in animals which acts upon the peptide linkage occurring in the protein substrate. Further definition of the enzyme includes an isoelectric point of 7 to 8, an optimal pH for activity of 6.8-7.5, and an optimal temperature range of 35 to 40 C.

In the purified state, the enzyme may be crystallized in the form of short prisms. The elementary composition of purified trypsin is as follows:

<table>
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<tr>
<th>Element</th>
<th>Percentage</th>
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<tr>
<td>Carbon</td>
<td>50.00%</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>6.86%</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>15.00%</td>
</tr>
<tr>
<td>Chlorine</td>
<td>2.85%</td>
</tr>
<tr>
<td>Sulphur</td>
<td>1.10%</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>0.00%</td>
</tr>
<tr>
<td>Ash</td>
<td>1.00%</td>
</tr>
<tr>
<td>Amino nitrogen as percentage of total nitrogen</td>
<td>9.30%</td>
</tr>
</tbody>
</table>
The approximate particle weight of the enzyme is 36,500, and its diffusion coefficient is .023 centimeters per day.

Although many enzymes show a great deal of specificity for substrate, trypsin acts upon a variety of substrates, the essential constituent being the peptide linkages. The rate of action, however, may be governed by the degree of extension of the peptide linkages in a given substrate. Thus, Haurowitz and his coworkers (20) found that globular proteins are attacked more rapidly after denaturation, whereas fibrous protein, i.e., fibrinogen and myosin are attacked at the same rate after denaturation as before. The experiments of Nakamura (26) indicate the variety of substrates as well as their cleavability.

Trypsin is an endopeptidase that cleaves the peptide bonds which are well removed from the terminal position of the peptide chain. The side chain requirements for trypsin action are the aminobutyl group found in lysine, or the guanidopropyl group found in arginine (15). Through cleavage of the peptide bonds, the complex protein substrate is broken down to the proteoses, peptones, polypeptides, and dipeptides (7).

The cleavage of protein chains by trypsin is influenced by many factors. The temperature and hydrogen ion concentrations are of utmost importance (15). Electrolytes (6), calcium (7) and hydroxyl ions (13), and cystine (19) have been found to increase the action of trypsin.
Conversely, many compounds have been shown to retard the action of trypsin, and in some instances the enzyme may be completely inactivated. Partial inhibition has been noted with many of the acid and basic dyes (4), while compounds such as cysteine, glutathione, 2, 3-dimercaptopropanol, and thiosalicylic acid will inactivate the enzyme completely (30). The mode of inactivation is unknown, but it is connected in some way with the appearance of sulfhydryl groups in the enzyme. This effect cannot be reversed by -S-S-compounds (30).

Another major group of substances which retard or prevent the action of trypsin is the animal antitrypsins. They include a specific serum globulin, a non-specific serum albumin, heparin, and egg white (13).

Spengler (1903) was among the early investigators who studied the effects of trypsin upon the tubercle bacilli. Pancreatin, actually a mixture of enzymes, was used as the digestive agent for clarifying sputum samples submitted for laboratory investigation. This substance proved suitable as the digestive agent and did not cause lysis nor distortion of the cells. Leibermeister (1905) also reported the absence of a bactericidal effect when pancreatic secretions were used. Loeffler (1913), however, submitted contradictory findings. He observed that when 5 mg of live tubercle bacilli were mixed with 20 mg of crude trypsin, and this mixture allowed to remain at 37 C for three days, the results were: 1) loss of pathogenicity for guinea pigs; 2) total
absence of growth when subcultured; 3) no alteration of acid fast stain.

It was not until 1926 that the study of the relation of the pancreas and pancreatic secretions to the destruction of the tubercle organism was again investigated. Robinovitch directed attention to the fact that the pancreas in tuberculous guinea pigs and cattle, in contrast to the liver, spleen and lungs, is completely free from infection. He maintained that guinea pigs which had received weekly injections of lipase had larger pancreases, were more immune, and escaped infection. On the other hand, guinea pigs with smaller pancreases in relation to body weight, succumbed to severe tuberculous infection. The agent responsible for this increased immunity seemed to be the lipase of the pancreas. Robinovitch also attributed immunity of the albino rat to tuberculosis to the large pancreas and large amount of lipase in the tissues. The organisms in the rat remained acid fast and were not infectious. Lipase sufficiency in these tissues, he maintained, sustained the tubercle bacilli in an attenuated form. Finally, he demonstrated that four hours after mixing viable tubercle bacilli with sterile, glycerol-pancreas extract, the organisms lost their property of acid fastness and were non-infectious for guinea pigs. The cause of these two alterations was attributed to a substance contained in the pancreas which hydrolyzed and then saponified the waxy or protective coating of the tubercle bacilli.
About this same time, Northup (1926) reported that living bacterial cells are resistant to the action of trypsin (36). Closely correlated with this was the work of Merrill (1936), who observed that Gram-negative organisms were resistant to the action of trypsin only while living (36). Thus, the Gram-negative bacteria differ from the Gram-positive organisms which resist the activity of the enzyme even after death. This resistance, he explained, may be the result of several possible factors such as cell membranes which are selectively impermeable to the enzymes; the presence of enzyme inhibitors in the cells; certain repellent forces between the cell membranes and the enzymes; or, the possibility that only denatured protein is susceptible to the action of trypsin.

Day and Gibbs (1930) again called attention to the human pancreas as one of the rarest seats of tuberculosis infection and demonstrated that when sterile extracts of pancreatic juice obtained from dogs were mixed for two hours with Mycobacterium tuberculosis, the organisms did not lose acid fastness, nor did lysis or dissolution occur. He accounted for the growth which occurred after four weeks in his subcultures to the fact that the bacilli frequently appeared in clumps, and the pancreatic secretions were not brought into contact with the surface of all of the cells. Berg (1932) also investigated the effects of pancreatic enzymes on tubercle bacilli. His contention was that the
cells undergo autolysis in vitro and liberate soluble digestion products. For example, if any portion of the fatty acid layer on the tubercle bacilli is digested by the enzyme, an increase in the acidity of the bacterial and enzyme suspension should be demonstrated. This was confirmed by measuring acidity of the bacterial suspensions by titration with N/20 NaOH at various intervals after the addition of the enzyme. The substance removed from the cells was not identified. Further support of his lysis concept was indicated by the phenomenon of swelling of the tubercle bacilli after exposure to trypsin. The volume of the cells increasing from 3.2 ml of cells at thirteen hours to 4.1 ml of cells in the course of seven days.

Reiser (1951), Madden (1952), and Seymour (1953) have reported on the use of this enzyme to digest suspected tuberculous sputum samples to facilitate microscopic examination of tubercle cells. Accuracy of 92 per cent was achieved in the detection of positive samples when 15 slides from 5 different sputum samples were investigated. No lysis of the cells was observed. Seymour found that 125 mg of trypsin dissolved in 12.5 ml Sorenson's phosphate buffer were sufficient to liquefy 8-10 ml of sputum. By adding 1-2 drops of caprylic alcohol to reduce surface tension and prevent foaming, which commonly occurs when protein solutions are agitated, he found that fifteen minutes agitation of the sputum sample at 57°C was sufficient for digestion. In addition,
if 0.5 to 1 ml of plasma was layered on the bottom of the centrifuge tube, no difficulty was encountered in fixing the smear and arresting the digestive process. Microscopic examination showed a reduction in the number of mature non-viable squamous cells which make up a considerable part of the sputum material. One interesting feature of this treatment of sputum with trypsin is that it does not affect malignant cells. This fact would aid considerably in the cytologic diagnosis of gastric cancer in which may be found large numbers of non-viable squamous and other cells which interfere with accurate diagnosis.

Although the man who originally conceived the idea of the utilization of trypsin for the treatment of tuberculosis was Bruning (1910), it was Keppleberg (1950) who initiated intrapleural use of trypsin specifically in tuberculosis for debridement of the pyogenic membrane of the empyema cavity. He achieved such successful results with eighteen patients, that large scale application of this method soon ensued. He instilled, intrapleurally, 150-200 units of trypsin and allowed it to remain in the chest cavity for five to six hours. The solution was removed and the cavity washed thoroughly with physiological saline. This procedure was repeated daily for five to six days. In the more severe cases, after a rest period of one week following the first series of instillations, the same procedure was repeated for an additional four to five days. After such treatment,
the tubercle organisms could no longer be detected in the aspirate. The only side reactions observed were a slight rise in temperature and sudden chill of very short duration, possibly as a result of the effects of the products of proteolysis or, a histamine reaction.

The group of Reiser, Roettig and Patton (1951) also successfully utilized trypsin to treat pulmonary tuberculosis with empyema. They instilled 500 mg of trypsin daily, and, in addition, administered Benadryl to suppress histamine-like reactions caused by the trypsin. Twenty-five to 50 mg of Benadryl were given intravenously followed by oral administration of 50-100 mg every three hours for three doses. Thereafter, no Benadryl was required.

In addition to the use of this enzyme for the treatment of tuberculosis empyema, these same men used trypsin in powder form successfully for the tryptic debridement of necrotic wounds and tissue, amputation stumps, subcutaneous hematomas, decubitus ulcers, and sloughing wounds after failure of skin grafts. When trypsin was applied to purulent and necrotic lesions, rapid lysis of the fibrinous strands and coagulum on the surface of the lesions occurred in a matter of minutes. It digested the viscid coating containing the desoxyribose nucleoprotein of the purulent exudate and, in addition, attacked the dead tissue in the lesion. There was an almost immediate outpouring of serum. The exudate became thin and serous, and fresh viable leucocytes appeared. Bac-
bacteria rapidly decreased in number and disappeared completely. A clear granulating pink surface remained. In the treatment of amputation stumps, a clean granulating area was obtained which was secondarily closed on the tenth day. Trypsin utilization frequently prevented higher amputation and markedly shortened the period of hospitalization.

In conditions such as subcutaneous hematomas, very marked success was achieved also by powder sprinkled over the clot. Liquefaction occurred within a matter of minutes so that adequate dressing and healing of the affected area rapidly occurred. In sloughing wounds, such as occur after failure of skin grafts, this enzyme again caused the removal of encrusted debris. A large collection of fluid again covered the ulcer. When this was removed, a fresh, red and vascular base was observed. Epithelization progressed rapidly and complete healing was obtained. Similar results were also achieved with decubitis ulcers, varicose ulcers and in the treatment of severe burns. (Struck, 1949).

Wet compresses were also used by Reiser in the treatment of gangrenous diabetic ulcers. Trypsin, 250 mg, was dissolved in Sorenson's phosphate buffer and the diluent plus new trypsin was administered every four hours. Structures were treated which were covered with a black slough of dead skin and which contained draining sinuses from which pus exuded. Staphylococci were the predominant organisms. Within forty-eight hours, the slough could be removed and
healthy living tissue exposed. In many cases, amputation was prevented.

Madden and Ravits (1952) recommended using trypsin as an adjunct in the treatment of indolent infected cutaneous ulcers. Thirteen patients with cutaneous ulcers were treated with trypsin, the object being to remove the adherent fibrino-purulent exudate from the ulcer surface in order that the formation of healthy granulation tissue be facilitated or proceed with further healing therapy. The crystalline trypsin was used in the form of wet compresses prepared by dissolving 250 mg in 25 ml of Sorenson's reagent, and also by direct application of the dry powder in the same concentration. Fresh preparations of the enzyme were made every three hours. With trypsin therapy administered in this manner, 11 of the 13 patients showed improvement.

Intravenous utilization of trypsin, on the other hand, was recently used by Innerfield, Angrist and Schwarz (1953). This group recommended a dose of 100,000 to 250,000 units of crystalline trypsin diluted in 250 cc of saline administered at the rate of 25-30 drops per minute, once or twice daily. A total of 6,456 infusions were performed on 538 patients. Alleviation of pain, erythema and tenderness were observed, and reduction in inflammation from acute thrombophlebitis, acute gouty arthritis and acute rheumatoid arthritis. The theory of trypsin action, postulated by them, is that trypsin plays a significant role in catalyzing enzymatic
sequences which augment the reaction of the host to injury, or that it activates naturally occurring enzymes which determine the rate at which the inflammatory reaction subsides.

Intravenous trypsin in the treatment of thrombotic phenomenon was extended considerably by Laufman and Roach (1953). In addition to noting improvement in acute thromophlebitis as above, they also determined that lysis of thrombi in animals could not be achieved unless some trypsin was infused prior to the formation of the thrombus and again afterwards. Furthermore, local pain, tenderness and redness occurred at the site of infusion. This was attributed to the fact that small concentrations of trypsin will convert prothrombin to thrombin and, to the low pH of the saline solution of trypsin. To alleviate this, trypsin was dissolved in larger quantities of saline and infused at a slower rate into an elevated arm with a 23 gauge needle. Contraindications listed were: bleeding tendency, congestive heart failure, liver failure, open intestinal lesions, and finally after four days instillation, the serum total protein dropped an average of 1.6 g per 100 ml. Most of this decrease occurred in the serum globulin level. No drop was perceptible in the fibrinogen level of the blood.

The anticoagulant capacity of trypsin is well known. Congestive heart failure, however, is attributed to cyanosis methemoglobinemia, since trypsinized red blood cells undergo a change in their oxygen carrying capacity. In addition,
the liver must produce sufficient trypsin inhibitors to protect viable cells and, it is obvious that any open pulmonary or ulcerative lesion could result in intense bleeding.

Since trypsin has the characteristics required for combatting altered pathology, particularly where avascular, proteinacious and necrotic conditions are prevalent, various methods were devised to demonstrate the action of trypsin upon Mycobacterium tuberculosis. Particular attention was centered upon pathogenicity, morphology, staining reactions, permeability and growth studies.
MATERIALS AND METHODS

Cultures and media employed

The following cultures were used in these experiments: *Mycobacterium tuberculosis* var. *bovis*, *Mycobacterium phlei*, *Mycobacterium smegmatis* and BCG. All were grown in Dubos Tween 80 liquid medium having the following composition:

\[
\begin{align*}
\text{KH}_2\text{PO}_4 & \quad \quad \quad \quad \quad 1.0 \text{ gm} \\
\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} & \quad \quad \quad 6.3 \text{ gm} \quad (2.5 \text{ gm anhydrous}) \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & \quad \quad \quad 0.01 \text{ gm} \quad (1 \text{ ml of a 1 per cent stock solution in distilled water}) \\
\text{CaCl}_2 & \quad \quad \quad 0.0005 \text{ gm} \quad (1 \text{ ml of a 0.05 per cent stock solution in distilled water}) \\
\text{ZnSO}_4 & \quad \quad \quad 0.0001 \text{ gm} \quad (1 \text{ ml of a 0.01 per cent stock solution in distilled water}) \\
\text{CuSO}_4 & \quad \quad \quad 0.0001 \text{ gm} \quad (1 \text{ ml of a 0.01 per cent stock solution in distilled water}) \\
\text{Ferric ammonium citrate} & \quad \quad \quad 0.05 \text{ gm} \quad (5 \text{ ml of a 1 per cent solution}) \\
\text{Asparagin} & \quad \quad \quad 2.0 \text{ gm} \\
\text{Enzymatic digest of casein} & \quad \quad \quad 0.5 \text{ gm} \quad (10 \text{ ml of a 5 per cent stock solution autoclaved in distilled water}) \\
\text{Tween 80} & \quad \quad \quad 0.5 \text{ ml} \\
\text{Glycerol} & \quad \quad \quad 1 \text{ per cent} \\
\text{Distilled water} & \quad \quad \quad 900 \text{ ml}
\end{align*}
\]

The mixture was shaken thoroughly, autoclaved at 120 °C for thirty minutes, and cooled to 45 °C. One hundred ml of
A 3-5 per cent solution of bovine plasma fraction V in saline were added after filtration. This substance was used to neutralize the fatty acids and other toxic substances produced by *Mycobacteria*. This medium was used within seven days. The final pH was 6.9-7.0. This medium was then distributed in the desired amount into tubes and flasks. All glassware was cleaned with Hemo-sol and tin covered rubber stoppers were used.

**Method of Trypsin Assay**

The test is based on the observation that trypsin inactivates thrombin. The activity of thrombin can be tested with a fibrinogen solution. These two reactions form the basis of the qualitative and quantitative assay for trypsin in the absence of other thrombin inactivators.

Thrombin substrate is prepared in Sorensen's phosphate buffer (pH 7) to an approximate concentration of 10 units per ml. The assays have been carried out with the Upjohn preparation of bovine origin.

A 3 per cent solution of fibrinogen indicator was prepared in distilled water. The fibrogen solution can be prepared by allowing the mixture to stand in an incubator at 37 C, with occasional stirring. When the solution is complete, the reagent is allowed to stand undisturbed for twelve to twenty hours (overnight) at 40 F. The solution for the test is obtained by the preparation of a carefully weighed
sample of crystalline trypsin (Armour)*, 100.0-150.0 mgs. Two-tenths mg is dissolved in 100 ml of Sorensen's phosphate buffer at pH 7. The concentration of trypsin is calculated and expressed in terms of the preparation as supplied.

All reagents are refrigerated at 40 F.

Quantitative Estimation

Into a clean dry test tube, deliver 3 ml of fibrinogen indicator. Place in water bath at 37 C.

Into a second clean dry test tube, deliver 1 ml of thrombin solution and bring to 37 C. At proper temperature add 1 ml of "unknown" solution and mix. Incubate mixture at 37 C for three minutes. Rapidly pour the contents of this "reaction tube" into the fibrinogen indicator and thence back into the trypsin-thrombin "reaction tube". Further agitation is undesirable, occasionally producing a flocculent precipitate which is difficult to read. Allow the tube to stand for five minutes at 37 C and read. A crystal clear fluid constitutes a positive test for trypsin; a frankly turbid or solid clot is read as negative; and a hazy fluid indicates a trace. The test is sensitive to a concentration of .05 mg of trypsin per ml in the solution being tested. The sensitivity of the test can be increased markedly by decreasing the concentration of the thrombin solu-

*Tryptar was supplied by the Armour Laboratory, Chicago, Illinois.
tion to the minimum required to clot the fibrinogen. However, the test becomes so sensitive that slight error in technique and traces of contaminants in the glassware render it unreliable for routine laboratory use.

**Quantitative Method**

**Standardization of the Thrombin Solution**

Proceed with fibrinogen indicator as described under the qualitative method. Prepare 10 test tubes each containing 1 ml of thrombin solution. By serial dilution, deliver into each thrombin containing tube 0.1 to 1.0 ml of the standard trypsin solution having made volume correction before addition of the trypsin such that the final volume is 2 ml. Incubate after trypsin addition for one minute at 37 C. Mix as previously described, allow to stand quietly and read after five minutes. The last crystal clear tube is taken as the end point representing total destruction of 1 ml of thrombin. (Fig. 1)

The "unknown" is handled in the same manner as the standard trypsin solution. For the most reliable results, the solutions are prepared daily; however, the tests can be duplicated within the limits of the sensitivity of the test over a forty-eight hour period if the reagents are refrigerated while not in use.

The separation between crystal clear fluid and opaque fluid is equivalent to 0.2 mg of crystalline trypsin (Armour)
Fig. 1. End point demonstration of trypsin assay method.
and, therefore, represents the sensitivity of the test. The sensitivity of the test can be increased by decreasing the thrombin concentration; however, the end point becomes difficult to read.

**Preparation of Trypsin Treated Cells**

An assay method for the determination of the direct action of varying amounts of trypsin on *Mycobacterium tuberculosis* was performed. Dubos medium, which does not inactivate trypsin, was used. The procedure for this assay was as follows: cultures of twenty-day Ravinal bovine strains of *Mycobacterium tuberculosis* were used. The cells were grown in 50 ml of Dubos medium contained in 250 ml Erlenmeyer flasks with tin capped rubber stoppers. The bacterial suspension was removed from the 250 ml flasks with sterile 50 ml pipettes and placed in Evelyn colorimeter tubes. The cells were allowed to settle out for two weeks at -10 C. The supernatant was removed and the cells were pooled in a 40 ml centrifuge tube. The final volume of cells after ninety-six hours at -10 C was approximately 10 ml. The pooled cells were washed 4 times in sterile physiological saline and divided into 2 equal 10 ml samples. Light transmission by each sample with the photoelectric colorimeter, using the 10 ml diaphragm and 540 filter, was 7 per cent. Both samples were again centrifuged, the supernatant was removed and replaced with 10 ml of sterile saline buffered with di-
phosphates to a pH of 7.5. The samples were then shaken, placed in two 50 ml Erlenmeyer flasks containing glass beads and a teflon covered magnet (5/16" x 1/4"). To one of the flasks was added 250 mg (250,000 Armour units) of Armour's crystallized trypsin dissolved in 10 ml of the buffered saline, and to the other flask 10 ml of buffered saline. Both samples were placed in the 4°C incubator and magnetically stirred using the number 20 setting on the magnetic mixer for twenty-four hours, after which time an additional 250 mg of trypsin in 5 ml buffered saline was added to the flask containing trypsin and 5 ml of buffered saline to the other flask. Both suspensions were magnetically stirred for an additional twenty-four hours. Both suspensions were then washed 4 times in sterile saline and the volume was adjusted to show a 10 per cent light transmission with the photoelectric colorimeter. The cells were then kept at -10°C until used. The cells prepared in the above manner were examined and subcultured as follows:

A. Subcultured into Petragnani, Dubos and Lowenstein medium.

B. Investigated for evidence of permeability or surface alterations using the basic fuchsin and methylene blue dyes.

C. Studied for the determination of any quantitative increase in the ingestion of the trypsin treated cells by rabbit macrophages.
D. Analyzed for any alteration of staining characteristics using the acid-fast, methylene blue and gram stain.

E. Injected into guinea pigs and mice for the determination of the LD/50.

The In vitro Method Employed for the Determination of Trypsin Activity Against Mycobacterium tuberculosis

Ten ml of a twenty day old culture of Ravinal strain of bovine Mycobacterium tuberculosis were standardized in the Evelyn colorimeter. The 10 ml diaphragm and the 540 filter were used. The standard sample showed a density of 58 per cent light transmission when compared with the control tube (10 ml of uninoculated medium). With a sterile 1 ml tip delivery pipette, 0.15 ml of the bacterial suspension was inoculated into a series of 10 tubes each containing 8 ml of Dubos medium. The tenth tube served as control, e.g., contained no trypsin. Another series of 9 tubes with a similar amount of medium was used as trypsin controls, that is, trypsin without the bacterial inoculum. The trypsin dilutions were prepared in such a way that the desired amount, expressed in milligrams (mg) would be contained in 2 ml of a Sorenson's standard phosphate buffer diluent, therefore bringing the amount in each tube to 10 ml. For example, starting with 250 mg vial of Armour's preparation
of crystallized tryptar, 2.5 ml of the sterile buffer were added giving a dilution of 100 mg/ml. Two ml of this dilution were added to the first tube giving a total volume of 10 ml (200 mg in 8 ml Dubos, plus 2 ml diluent = 20/mg/ml). Decreasing amounts were prepared in the same manner. Trypsin in amounts of 200, 150, 100, 50, 25, 10, 5, 1 and 0.2 mg were used. The tubes containing the same amounts of trypsin but without the inoculum were used as controls in order to check (1) the sterility of the tryptar preparations, (2) to adjust accurately the colorimetric readings, and (3) to determine if trypsin was inactivated in Dubos medium. After four weeks, subcultures were prepared from all tubes into Dubos and Petragnani medium.

Photoelectric Measurements of Dye Uptake by Trypsin Treated and Non-Trypsin Treated Organisms

In order to measure any change in dye absorption resulting from exposure of the organism to trypsin, it was necessary to prepare standard reference curves. The procedure for this was as follows: A stock solution of the dye basic fuchsin was prepared by dissolving 0.1 gm of dye in 1000 ml of distilled water. Dilutions of the dye were made from this stock (100,000, 200,000, 400,000, 600,000,
Figure 2 was deleted from the text.
800,000 and 1,000,000), and maximum light absorption was determined with the spectrophotometer (Model B) using the different wave lengths of light and each of the dilutions prepared above. The readings of maximum light absorption obtained with the dilutions of dye ranging from 200,000 to 1,000,000 at the different wave lengths are shown in Fig. 3. It is evident that the widest range of light absorption, 0.40 to 1.20 per cent, was obtained with dilutions of dye ranging from 200,000 to 600,000 at 550 mu. Therefore, the wave length used was 550 mu and the dye dilution 200,000.

The final procedure used for this experiment was as follows. Three ml of the standardized suspension of trypsin treated cells (t.t.c.) and non-trypsin treated cells (n.t.c.) were added to 8 ml of the 200,000 dilution of basic fuchsin and placed in a 12 ml centrifuge tube. Each mixture was shaken and allowed to remain thirty minutes, one hour, and three hours, respectively, in the 37 C water bath. The tubes were then centrifuged and spectrophotometric readings taken of the supernatants.

In order to determine whether dye uptake was intracellular or merely adsorption, the method (8) of Bloch was used, with slight modifications. The cells to be treated had been carried through 3 successive passages in mice to insure virulence and were treated in the same manner as above except that a fourteen day culture was used. Both the t.t.c. and
Fig. 3 Light Absorption Using Different Dilutions of Dye
n.t.c. were washed three times in distilled water. To Thunberg tubes was then added 0.5 ml of M/15 phosphate buffer, 0.5 ml of a 1 per cent dextrose solution and 0.5 ml of 1:20,000 dilution of methylene blue. To the Thunberg cups was then added 0.5 ml of the t.t.c. and n.t.c. The tubes were immediately immersed in ice water and evacuated for four minutes. The cells were then dumped into the substrate, buffer, dye mixture and placed in the 37 C water bath. The decolorization time was then recorded.

Methods Employed for the Investigation of the Phagocytic Capacity of Rabbit Macrophages Using Trypsin Treated and Non-Trypsin Treated Cells

Macrophages cultured from rabbit spleen were used. Various methods were attempted to study phagocytosis of tubercle bacilli by macrophages. Supravital staining was satisfactory except for the fact that with normal cells penetration of the dye occurred slowly, therefore, the dye and detergent used had to be of considerable strength, hence cells treated in this manner were considerably altered. Therefore, the preparations stained to show the macrophages and tubercle organisms proved to be the most satisfactory. This procedure was as follows. The washed treated and non-treated cells were suspended in Earl's (41) solution to which penicillin had been added (100 units/ml). Five-tenths ml of the
treated and of the non-treated cell suspension were diluted with five ml of Earl's solution. Utilizing a 1 ml tip delivery pipette, 1 drop of the mixture of tubercle cells plus Earl's medium and 2 drops of normal rabbit serum were added to each tissue culture preparation (41). The cultures were allowed to incubate at 37 C for forty-five minutes, after which time the fluid was removed, the cover slip containing the tubercle cells and macrophages was fixed in absolute methyl alcohol for ten minutes and immediately dried in the 37 C incubator for thirty minutes. The following staining procedure which is a slight modification of that of Suter was then applied (40). The cover slips were immersed in Ziehl-Neelsen stain at room temperature for twelve minutes, washed in distilled water, decolorized by immersing in 3 per cent acid alcohol for approximately three to four seconds and counterstained for eight minutes with methylene blue. The cover slips were then washed and dried in the 37 C incubator and were mounted on microscopic slides with balsam and observed. The tubercle bacilli within the macrophage appeared as a reddish pink elongated structure against a background of dark blue nuclei and the light staining blue of the cytoplasm. In every case, 45 tissue culture slides were prepared, 15 each for n.t.c., t.t.c., and controls. One hundred macrophages were counted on each slide. The numbers of phagocytic cells with and without tubercle organisms were recorded and also the number of bacilli within the individual
phagocytic cells.

The best contrast and photographs were obtained by using a 12X compensatory eyepiece, the 90X apochromat objective (N.A.I. 30) and the achromat Abbey condenser (N.A.I. 25). A field image of 22 centimeters was utilized, and by employing filter numbers 16, 17, 18 and 22 an approximate magnification of 2000X was obtained. The photographs are presented in Figures 12 and 13.

Trypsin Potentiation of Antibiotic Activity

The possibility of trypsin potentiation or decrease of antibiotic activity was investigated. Dihydrostreptomycin, Aureomycin, Penicillin and Aerosporin were selected for study.

A pooled eighteen day old culture of Mycobacterium tuberculosis var. bovis was adjusted colorimetrically to 5 percent density and served as the inoculum. From this suspension 0.3 ml was pipetted into 90 Evelyn colorimeter tubes containing 8 ml of freshly prepared Dubos medium. The inoculated tubes were placed in the 37 C incubator and allowed to grow for seven days. On the seventh day, the following concentrations of Penicillin and Aerosporin were prepared in distilled water and added in 2 ml amounts to 40 of the tubes resulting in 200,000, 150,000, 100,000, 50,000, 25,000, 10,000, 5,000, 1,000, 500 and 100 units per tube. To an
additional tubes, the same concentrations of Aureomycin and Streptomycin were added in 1 ml amounts, and additional units as low as 10 to 1 were also included. In addition, 1 ml of 10,000 units of trypsin, which does not inhibit growth of *Mycobacterium tuberculosis*, was also added. All densities were recorded and the tubes were again incubated at 37 C. Trypsin assays were completed previous to the above experiments to determine how many units of trypsin/ml would be inhibitory to the growth of the tubercle bacillus. This was found, as before, to be between 25,000 (25 mg) and 10,000 (10 mg) units. Controls containing 10,000 units of trypsin in 2 ml and another containing 2 ml of sterile distilled water were added to 8 ml of inoculated Dubos medium.

**Determination of the 50 per cent Survival Time in Guinea Pigs and Mice Using Trypsin Treated and Untreated Mycobacterium tuberculosis**

White, female Swiss mice weighing 20-25 grams, 10-12 weeks of age were used. Intranasal inoculations of trypsin were performed with a blunt twenty gauge needle while the mice were under ether anaesthesia. The intravenous injections of the bacterial suspensions were given in one of the four lateral tail veins using the 1 ml tuberculin syringe and a twenty-four gauge needle.

Three twenty day old cultures of *Mycobacterium tuber-*
-32-

Mycobacterium tuberculosis grown in 10 ml Dubos medium were adjusted colorimetrically to a density of 50 per cent. The three samples, after being standardized colorimetrically, were used in the following manner. One tenth ml was removed from one sample and injected intravenously into 10 mice for the determination of the 50 per cent survival time. Another of the samples was trypsin treated as mentioned above and a similar amount was injected intravenously into 10 additional mice.

Investigations were also undertaken with guinea pigs to determine the virulence of treated and non-treated tubercle bacilli. A series of 16 guinea pigs were injected. Eight guinea pigs were injected subcutaneously with 0.15 ml of the non-treated cell suspension and eight additional animals were injected in the same manner with the enzyme treated cell suspension.

Procedure for the Evaluation of the Therapeutic Action of Trypsin

For this determination 20 mice were infected with normal cells in a similar manner. Ten received 5 mg of trypsin intranasally contained in 0.05 ml sterile phosphate buffer every 48 hours after the initial 0.1 ml intravenous inoculation of the bacterial suspension. With the remaining 10 mice the infection was allowed to progress two weeks after which time they also received 5 mg trypsin intranasally in
0.05 ml amounts. Controls for the above experiment were determined as follows. Thirty mice in groups of 5 were allowed to inhale the following concentrations of trypsin/ml: 20, 10, 5, 1, 0.5, and 0.1 mg. The concentration of trypsin chosen for therapeutic use was the highest concentration which all five mice could tolerate. This proved to be 5 mg/ml. Furthermore, to determine whether the enzyme entered the lung, an autopsy was performed on the mice which survived 45 minutes and 48 hours after intranasal inhalation. Intense hemorrhagic areas were evident in the mice that succumbed, e.g., received the 20 mg/ml. The remainder showed similar pathology but to a lesser degree. After forty-eight hours the lungs of all surviving animals appeared normal.

**Determination of the hypersensitive condition utilizing enzyme treated cells**

Skin hypersensitivity tests using tuberculin, and trypsin treated cells were also completed with the same animals used above for virulence tests. The infection previously produced in the 16 guinea pigs was allowed to progress for a period of four weeks. Four guinea pigs which had been injected with the enzyme treated cells were given 0.1 ml of trypsin treated cells intradermally and the other four 0.1 ml of P.P.D. intradermally. The same procedure was repeated with the guinea pigs which had been infected with
normal cells. Normal guinea pigs served as controls. The hypersensitive phenomenon was then observed with regard to time of appearance and characteristic pathology.

The final samples of non-treated tubercle organisms were washed three times in sterile distilled water and injected intravenously into mice as above to determine the 50 percent survival time. This experiment was included to determine if the washing of the organisms alone would reduce the survival time.
EXPERIMENTAL RESULTS

Effect of Trypsin on the Growth of Mycobacterium tuberculosis

Various concentrations of trypsin were tested for bacteriostatic and bactericidal action on Mycobacterium tuberculosis. The enzyme was dissolved in Sorenson's phosphate buffer and added to 8 cc of Dubos medium to give the following concentrations: 200, 150, 100, 50, 25, 10, 5, 1 and .2 mgs. The inoculum consisted of 0.15 ml of a standardized suspension of the organism.

A comparison of the growth in the presence of the enzyme may be had from comparing the data in Figure 4 obtained by turbidometric measurements of test and control samples at various times during the incubation period. The typical growth curve is observed in the absence of trypsin. However, no growth occurred in concentrations above 25 mgs (2.5 mg/ml). Subcultures prepared from the 4 highest concentrations of trypsin to Petragnani and Dubos media after four weeks also remained negative showing that these amounts of trypsin were bactericidal, while the 2.5 mg/ml amount was only bacteriostatic. Microscopic examination of the organisms previously exposed to high trypsin concentrations showed that the organisms had maintained their typical morphology. They also remained acid fast. The sediment from such cultures also differed from control cultures without trypsin, being more
Fig. 4 The Effect of Various Concentrations of Trypsin upon \textit{Mycobacterium Tuberculosis}

![Graph showing the effect of various concentrations of trypsin on \textit{Mycobacterium Tuberculosis}. Controls and trypsin present 200-25 mg are compared over a period of days.]
granular and flaky instead of stringy and viscid.

No bactericidal or bacteriostatic effect could be demonstrated in cultures containing 10, 5, 1 and 0.2 mgs of trypsin. Data from these experiments are presented in Figure 5, and the similarity may be demonstrated by comparison with the control cultures, Figure 4. Thus, in the presence of the lower concentrations of trypsin, the Mycobacteria followed the typical growth cycle. In addition, there was no growth stimulation by trypsin in the lower concentrations as reported by others (18), nor was there any increase or decrease in cell volume, nor alteration in morphology and acid-fastness.

From these experiments, it was evident that trypsin was bactericidal for Mycobacterium tuberculosis in concentrations of 50 mgs/ml and above, and the growth of the organisms was inhibited by 2.5 mg/ml. Lower concentrations in Dubos medium were used without any demonstrable effect.

Characteristics of Mycobacterium tuberculosis after Trypsin Treatment

Apart from the exposure of the bacilli to trypsin in Dubos medium, followed by subculture and acid fast studies four weeks later, additional information was required concerning the tryptogenic effect upon the tubercle organisms. Therefore, 2 samples of standardized suspensions of organ-
Fig. 5 The Effect of Various Concentrations of Trypsin Upon *Mycobacterium Tuberculosis*

- Circle = 10 mg
- Circle with hole = 5 mg
- X = 1 mg
- Dot = 0.2 mg

Per Cent Light Transmission (Density)

Days: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
isms were washed and treated with trypsin as described previously. Chymotrypsin was also used as a proteolytic agent.

Subculture and acid fast studies in this case were made forty-eight hours after exposure. Furthermore, possible cell wall alterations were investigated. Lowenstein and Petragnani media with and without malachite green, were used as additional media. After 48 hours exposure, the cells treated with trypsin and chymotrypsin maintained their ability to resist decolorization and retain the cell wall stain. In addition, six weeks after the preparation of subcultures from both sets of treated cells, growth was still absent on Petragnani slants and in Lowenstein and Dubos media. In addition to the absence of growth observed above, acid fast bacilli could not be demonstrated on Petragnani slants. However, when Petragnani medium without malachite green was used as the subculture medium, acid fast rods from the chymotrypsin treated sample could be demonstrated but not from the trypsin treated sample. The results are illustrated in Table I.

Potentiation of Antibiotic Activity by Trypsin

Since previous investigations have determined that 10 mg of trypsin are not bactericidal for mycobacteria and, as we will be shown later, metabolic changes are apparent after exposure of the bacilli to this enzyme, the possibility occurred that the above concentration of trypsin, although
**TABLE I**

**Morphology, Acid Fast Staining and Growth Characteristics of *Mycobacterium tuberculosis* on Different Media Before and After Trypsin Treatment**

<table>
<thead>
<tr>
<th></th>
<th>Before Enzyme Treatment</th>
<th>After Enzyme Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Fast Staining</td>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td>Retention of Cell Wall Stain</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth on Dubos Medium</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Petragnani Medium</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Petragnani Medium (Malachite green absent)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lowenstein Medium</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Absence of acid fast rods
not bactericidal, may play a role in the potentiation of antibiotic activity. Therefore, attempts were made to determine the potentiation of the activity of Penicillin, Aerosporin, Aureomycin and Dihydrostreptomycin.

TABLE II

Trypsin Potentiation of Antibiotic Activity

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Minimum number of units required for growth inhibition</th>
<th>Antibiotic plus trypsin (10,000 units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>10,000</td>
<td>10,000</td>
</tr>
<tr>
<td>Aerosporin</td>
<td>200,000</td>
<td>200,000</td>
</tr>
<tr>
<td>Aureomycin</td>
<td>10,000</td>
<td>1,000</td>
</tr>
<tr>
<td>Dihydrostreptomycin</td>
<td>100</td>
<td>1</td>
</tr>
</tbody>
</table>

Measurements of the degree of growth turbidometrically indicate that the presence of 10,000 units of trypsin had no effect on the minimum concentrations of Penicillin and Aerosporin required to inhibit the tubercle bacilli. However, a similar amount of the enzyme increased the action of Aureomycin and Dihydrostreptomycin ten-fold and 100-fold, respectively.
Surface Alterations of Enzyme Treated Mycobacterium tuberculosis

Surface phenomena and permeability alterations have been shown by Bloch to play a dominant role in the pathogenesis of the tubercle organisms (2,8). By extraction of virulent tubercle organisms with petroleum ether, he has removed a lipid material from the surface of the cell, which controls the release of the intracellular enzymes. The enzymatic dehydrogenase activity of the extracted cells may be demonstrated by the Thunberg-methylene blue method.*

It is evident from Figure 6 that trypsin treated cells "take up" much more dye (1.20 to 0.40 light absorbed), and that this uptake is complete within thirty minutes, whereas, in the non-treated cells the dye uptake is less and gradually increases as the incubation period is prolonged (1.20 to .98 light absorption after thirty minutes and .98 to .75 light absorption after one hour). Such a gradual penetration indicates a slow permeation of the dye into the cells or normal tuberculous organisms. These results indicate that trypsin produced some alteration of the surface of the tubercle bacilli which allowed the cells to adsorb more dye on the surface, or enabled the dye to permeate the cell more readily. The next step, therefore, was to use the method of Bloch, as mentioned above, for the determination of a permeability change. Methylene blue in the presence of an ap-
Fig. 6 Uptake of Dye by Trypsin Treated and Non-treated *Mycobacterium Tuberculosis*
propriate substrate such as 1 per cent glucose was decolorized by suspensions of avirulent or saprophytic mycobacteria. The saprophytic bacteria, for example such as *Mycobacterium phlei* and *Mycobacterium smegmatis*, decolorized methylene blue within three to five minutes. The avirulent strains such as BCG, on the other hand, required fifteen minutes, whereas, young virulent organisms did not decolorize the dye. The petroleum ether extracted virulent bacilli, on the other hand, decolorized methylene blue in a matter of minutes, indicating a removal of some surface component which altered the permeability of the tubercle cell. In addition, the extracted tubercle organisms take on other characteristics of avirulent cells after removal of lipid material. They no longer inhibit the migration of leukocytes after phagocytosis and they also exhibited a reduced state of pathogenicity. Because of the changes produced by trypsin in the previous experiments, the ability of the enzyme to affect this polysaccharide-lipid layer was examined. Thus, the time required for the decolorization of the mycobacteria, treated with both trypsin and chymotrypsin, was determined. The trypsin treated virulent mycobacteria decolorized methylene blue within thirty minutes. Fifteen minutes were required for the bacilli treated with chymotrypsin. The non-treated virulent organisms failed to decolorize within two hours at which time the test was concluded.
A substance capable of the rapid reduction of mycobacteria has been shown previously to be present in supernatants of ground tubercle bacilli. The enzymatic nature of this phenomenon is shown by the fact that it is inactivated at 56°C in four to five minutes and shows optimum activity at 37-40°C, being inactive at 20 or 45°C. It would seem from the above experiments, that trypsin effects an "extraction" of this substance by the alteration of permeability just described.

Phagocytosis of Normal and Trypsin Treated Mycobacteria by Rabbit Macrophages in Tissue Culture

The mycobacteria exposed to trypsin as mentioned above and then added to tissue culture preparations of rabbit spleen in which macrophages were actively proliferating, exhibited the following characteristics:

TABLE III
Phagocytosis of Trypsin Treated and Non-treated Mycobacterium tuberculosis by Tissue Culture Macrophages

<table>
<thead>
<tr>
<th>Percentage of Macrophages showing phagocytosis</th>
<th>Trypsin Treated Cells</th>
<th>Non-Tryptsin Treated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of bacilli within individual macrophages</td>
<td>0-2 3-5 6-10 10</td>
<td>0-2 3-5 6-10 10</td>
</tr>
<tr>
<td></td>
<td>12 15 34 39</td>
<td>57 30 8 5</td>
</tr>
</tbody>
</table>
First, more of the macrophages contained trypsin treated cells (t.t.c.) than non-treated cells (n.t.c.). Secondly, it was very common to observe macrophages containing more than 10 trypsin treated mycobacteria per macrophage and very few macrophages contained less than 3-5 per cell. In contrast, with the cells which were not exposed to trypsin, there were many macrophages observed without any engulfed organisms. Furthermore, very few macrophages had phagocytized as many as 10 microorganisms per cell and the majority contained only 1-2 cells per macrophage. Therefore, trypsin treatment of the cells resulted in an increase in the number of bacilli ingested.

**TABLE IV**

Death and Survival of Tissue Culture Preparations After 48 Hours Incubation in the Presence of Trypsin Treated and Non-Treated Cells

<table>
<thead>
<tr>
<th>EXPERIMENT</th>
<th>TTC</th>
<th>NTC</th>
<th>CONT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L D</td>
<td>L D</td>
<td>L D</td>
</tr>
<tr>
<td>I</td>
<td>2 13</td>
<td>1 14</td>
<td>14 1</td>
</tr>
<tr>
<td>II</td>
<td>3 12</td>
<td>0 15</td>
<td>15 0</td>
</tr>
<tr>
<td>III</td>
<td>2 13</td>
<td>5 10</td>
<td>14 1</td>
</tr>
<tr>
<td>IV</td>
<td>0 15</td>
<td>3 12</td>
<td>12 3</td>
</tr>
<tr>
<td>V</td>
<td>1 14</td>
<td>5 10</td>
<td>10 5</td>
</tr>
</tbody>
</table>
To examine the relative toxicity of treated and untreated tubercle bacilli for the macrophages, the t.t.c. and n.t.c. were allowed to remain in contact with the macrophages for forty-eight hours, at which time the cultures were observed for evidence of macrophage death or destruction. The results of 5 such experiments are included in Table IV. Destruction of approximately 86 per cent of the tissue culture macrophages was noted with t.t.c. and approximately 80 per cent destruction with the n.t.c. At the same time, 90 per cent of the macrophages of the control cultures survived. Photographs showing macrophages containing t.t.c. and n.t.c. are shown in Figures 12 and 13.

Chemotherapeutic Evaluation of Trypsin

To note further if the enzyme alteration of the mycobacteria would result in reduction of pathogenicity, ten mice were injected intravenously with the treated standardized bacterial suspension. Thirty additional mice were injected with normal cells in the same manner. Ten of these served as controls; 10 others were treated with trypsin intranasally every forty-eight hours; and the remaining 10 were given the same treatment with trypsin starting ten days after inoculation. An additional 10 mice were injected intravenously with washed non-treated mycobacteria in order to determine if the mere mechanical washing with sterile
saline reduced the pathogenicity. In all animals, the 50 per cent end point was determined and lung pathology was observed. Results are recorded in Table V.

It is evident that the survival rate of 50 per cent of the control mice was approximately fourteen days (Figure 7), while the survival rate of mice treated intranasally with 4 mg of trypsin every forty-eight hours was twenty-six days (Figure 8). On the other hand, if the infection was allowed to progress for ten days before trypsin treatment was initiated, the survival rate of the mice was twenty-one days (Figure 9). When the organisms were washed three times in sterile distilled water but not treated with trypsin, the 50 per cent end point was similar to the above controls. When trypsin treated organisms were used, the 50 per cent survival rate was increased to thirty days (Figure 10). The data concerning survival day by day during the experiment are also presented in Table V.

The lung pathology of all animals was observed and recorded as 4+, 3+, 2+, and 1+. (Table V). The basis upon which these numbers were interpreted was area of the lung covered with tubercles, their size, and whether the tubercles were confluent (touching each other) or whether they were discrete. In other words, the 4+ indicates more than 50 per cent of the organ covered with large confluent tubercles; 3+, about 25-50 per cent of the organ covered with fairly large confluent tubercles; 2+ indicates less than
25 per cent of the organ covered with small discrete tubercles; and 1+, less than 10 per cent of the organ covered with small discrete tubercles. In the group of mice which received trypsin intravenously every forty-eight hours after inoculation, the 50 per cent survival time was extended twelve days beyond the controls (Figures 7 and 8), and those which had survived beyond this time showed only 10 per cent lung involvement in contrast to the lungs of control mice in which approximately 50 per cent of the organs contained large confluent tubercles (Table V(a) and (b)).

Furthermore, when the tuberculous infection was allowed to progress for ten days before intravenous trypsin treatment was administered, the survival rate was extended only seven days over that of the control mice (Figures 9 and 7), and the disease process in the lungs was similar in extent and severity to that of the controls described above (Table V(c)). Furthermore, the lung pathology of the 10 mice which received the trypsin treated cells intravenously (Table V(c)) was only slightly different from those of the controls (Table V(a)), although the survival rate was 16 days longer (Figure 10). Approximately 25 to 50 per cent of the lungs of all animals were covered with moderately large confluent tubercles. Finally, the lung pathology of the mice infected with non-treated but washed mycobacteria was similar to that of the control mice.

In addition to the therapeutic evaluation of trypsin
TABLE V

The Effect of Trypsin Treatment on the Survival and Lung Pathology of Mice Infected with Mycobacterium tuberculosis

<table>
<thead>
<tr>
<th>(a) CONTROL MICE</th>
<th>(b) TRYPsin TREATED MICE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day of Death</strong></td>
<td><strong>No. dead</strong></td>
</tr>
<tr>
<td>8</td>
<td>10/1</td>
</tr>
<tr>
<td>10</td>
<td>10/2</td>
</tr>
<tr>
<td>12</td>
<td>10/3</td>
</tr>
<tr>
<td>14</td>
<td>10/4</td>
</tr>
<tr>
<td>15</td>
<td>10/6</td>
</tr>
<tr>
<td>15</td>
<td>10/7</td>
</tr>
<tr>
<td>17</td>
<td>10/8</td>
</tr>
<tr>
<td>18</td>
<td>10/9</td>
</tr>
<tr>
<td>22</td>
<td>10/10</td>
</tr>
</tbody>
</table>

(c) Mice Trypsin Treated after Prolonged Infection

<table>
<thead>
<tr>
<th><strong>Day of Death</strong></th>
<th><strong>No. dead</strong></th>
<th><strong>Tubercle formation</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>10/1</td>
<td>2+</td>
</tr>
<tr>
<td>16</td>
<td>10/2</td>
<td>2+</td>
</tr>
<tr>
<td>18</td>
<td>10/4</td>
<td>2+</td>
</tr>
<tr>
<td>21</td>
<td>10/5</td>
<td>3+</td>
</tr>
<tr>
<td>22</td>
<td>10/6</td>
<td>3+</td>
</tr>
<tr>
<td>25</td>
<td>10/7</td>
<td>3+</td>
</tr>
<tr>
<td>26</td>
<td>10/8</td>
<td>3+</td>
</tr>
<tr>
<td>28</td>
<td>10/9</td>
<td>4+</td>
</tr>
<tr>
<td>29</td>
<td>10/10</td>
<td>4+</td>
</tr>
</tbody>
</table>

(d) Mice Infected with Trypsin Treated Cells

<table>
<thead>
<tr>
<th><strong>Day of Death</strong></th>
<th><strong>No. dead</strong></th>
<th><strong>Tubercle formation</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10/1</td>
<td>2+</td>
</tr>
<tr>
<td>18</td>
<td>10/2</td>
<td>2+</td>
</tr>
<tr>
<td>23</td>
<td>10/3</td>
<td>4+</td>
</tr>
<tr>
<td>25</td>
<td>10/4</td>
<td>4+</td>
</tr>
<tr>
<td>33</td>
<td>10/5</td>
<td>4+</td>
</tr>
<tr>
<td>35</td>
<td>10/6</td>
<td>4+</td>
</tr>
<tr>
<td>38</td>
<td>10/7</td>
<td>4+</td>
</tr>
<tr>
<td>43</td>
<td>10/8</td>
<td>4+</td>
</tr>
<tr>
<td>51</td>
<td>10/9</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>10/10</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 7 Survival Time of Mice Infected With Mycobacterium Tuberculosis

Fig. 8 Survival Time of Infected Mice Treated With Trypsin Intranasally

4 Remaining Mice were killed at this date and lungs examined.
Fig. 9 Survival Time of Mice Treated With Trypsin After Prolonged Infection

Fig. 10 Survival Time of Mice Infected With Trypsin Treated Mycobacterium Tuberculosis
in mice, the same procedure was repeated with guinea pigs. However, in addition to measurements of survival time, and the observation of the disease process, hypersensitivity tests were also performed.

Sixteen guinea pigs were inoculated, 8 of which received subcutaneously 0.1 ml of the standardized enzyme treated bacterial suspension, and the remainder were injected similarly with the standardized non-treated bacterial suspension. Four guinea pigs were used as controls.

The tuberculous infection was allowed to progress for a period of four weeks in all 16 animals. Following this period, 8 guinea pigs which were infected with the trypsin treated cells were divided into two groups of 4, and 0.1 ml of P.P.D. was injected intradermally into one set of 4. Similarly, 0.1 ml of trypsin treated cells was injected into the other 4 animals.

The guinea pigs which had received the P.P.D. showed a moderate edema and redness at the site of the injection within twenty-four hours, which became very marked at the end of forty-eight hours. This redness persisted for an additional forty-eight hours and then disappeared. However, a different reaction was observed in those animals which received 0.1 ml of trypsin treated cells intradermally. Moderate edema appeared within 24 hours at the site of inoculation, followed by marked redness in forty-eight hours. The area involved became circumscribed and localized and the redness
disappeared within four days followed by a gray small pustule. This gradually became larger, until after approximately the ninth day, it had reached a diameter of three-fourths inches (Figure 11). About the twelfth day, the pustule broke open and a very viscous, grayish, pyogenic material exuded. This pustule became encrusted on the sixteenth day and almost completely healed over on the twenty-fifth day. Subculture of the pyogenic material resulted in a pure culture of acid fast mycobacteria.

Thus, when P.P.D. was used in the guinea pigs infected with trypsin treated cells, the typical hypersensitive condition appeared within 24 hours. When challenged with the same organism intradermally, however, a different response ensued, which included redness, pustule, ulcerous pustule, encrustation and healing within twenty-five days.

Two of the non-infected guinea pigs used as controls exhibited no pathology when 0.1 ml of trypsin treated cells and P.P.D. were injected intradermally. The guinea pigs which were infected with the trypsin treated cells and which received no further treatment, succumbed within the fourteenth to fifteenth week.

The 8 guinea pigs which were infected with the non-trypsin treated cells were also divided into groups of 4, and .1 ml of P.P.D. was injected intradermally into 1 group. One-tenth ml of trypsin treated cells was injected into the other group. Guinea pigs which received the P.P.D. intra-
dermally exhibited the same degree of edema and redness which appeared and disappeared at approximately the same time as the guinea pigs which were infected with the trypsin treated cells. Thus, no alteration occurred, both with regard to time of appearance and pathology of the hypersensitive reaction in animals infected with trypsin treated cells and non-trypsin treated cells.

The guinea pigs infected with non-trypsin treated cells which received 0.1 ml of trypsin treated cells intradermally, reacted in a manner similar to those infected with trypsin treated cells. However, the pustule was not as large (one-fourth inch) nor did it break open. In addition, the entire response with complete healing of the area injected took only about six days.

No altered pathology was observed in the two control guinea pigs after the intradermal injection of 0.1 ml of P.P.D. and trypsin treated cells. The animals which were infected with the non-treated trypsin cells succumbed within the seventh to eighth week.
Fig. 11. Localization of trypsin treated tubercle bacilli after intradermal injection.
Fig. 12. Phagocytosis of trypsin treated tubercle bacilli by rabbit macrophages.
Fig. 13. Phagocytosis of untreated tubercle bacilli.
DISCUSSION

The reason for this investigation of the effects of trypsin upon *Mycobacterium tuberculosis* was primarily the encouraging results achieved when trypsin was used intrapleurally as a debridement in patients with chronic tuberculous empyema. Microscope examination of the aspirate after intrapleural instillation showed the absence of tubercle organisms (34). Thus, the enzyme had either destroyed both the avascular tubercle and retained enough activity to effect destruction of the organism directly, or, it had destroyed the tubercle and exposed the bacteria to the phagocytic and humoral defenses. Direct evidence of the latter was obtained by Reiser and his group (34). Investigations of the intrapleural aspirate after trypsin lavage showed a marked drop in the fluid viscosity due to an increase in the serous component of the fluid. The reduced viscosity was due to the lysis of the nucleoprotein slime adherent to the pleural surface and the histamine liberating properties of trypsin. The process was also aided by the lysis of floating fibrin particles contained in the thrombogetic substances of the tubercle which were released into the pleural space.

Before proceeding further with the discussion of this problem, however, it would be advantageous to determine what constitutes the potential basis for pathogenicity of the tubercle bacilli. This can best be analyzed by a
consideration of the factors which contribute to such pathogenicity. First of all, there is the possibility that extracellular components, possibly of an enzymic nature, are elaborated by the host which may be neutralized or destroyed by the virulent tubercle bacilli. For example, high antitryptic titres are frequently observed in patients with active tuberculosis. Perhaps, if the trypsin level of the blood could be increased, so that it would not be neutralized or destroyed by the antitrypsin, the destructive capacity of this in vivo mechanism would be enhanced. Furthermore, increased resistance to tuberculosis has been observed in humans with a high serum lipase content (3). In favorable cases of tuberculosis, for example, the lipase content of the serum is high whereas in patients whose progress is downward the lipase level is low. In addition, it has been shown that in mice which have good innate resistance, the tissues are also rich in phosphatase and lecithinase. These enzymes were observed to be active upon the phosphatides of the tubercle bacilli.

Secondly, virulent organisms may possess substances capable of interference with the normal defense mechanism of the body. Such substances could interfere with the capacity of the phagocytes to destroy the organisms. Conversely, if we could detect interference with the multiplication and survival of the enzyme treated bacilli within the phagocytic cells, it would contribute to the important
feature of acquired immunity. It is well known, however, that the tubercle cell does overcome the primary defense mechanism of the host by actively proliferating within the phagocytic cells and ultimately destroying them. It is also known that these cells are ingested whether they are virulent or avirulent. The latter, however, are killed within the phagocytic cells while the former destroy this protective cell. Whether this destruction is caused by a substance elaborated by the organism after ingestion, or whether it is mainly due to proliferation of the organism within the phagocytes, remains to be determined. It is known, however, that proteolytic enzymes of a trypsin-like nature are found in polymorphonuclear cells and pepsin-like ferments are found in the macrophages (29). Whether these enzymes are responsible for the destruction of the avirulent bacilli, yet do not affect the virulent organisms, also remains to be elucidated. In addition, since we have demonstrated permeability changes, might not the release of substances other than dehydrogenases from the treated cells cause a lowering of the surface tension of the macrophage enough to cause formation of pseudopods which engulf the organisms (1). Differences in electric potential may also account for the greater attraction between the treated tubercle cells and the phagocytic cells (1). Whatever the reason, the tubercle do not discourage ingestion.

The quantitative difference and the rapidity with which
the number of organisms were ingested after enzyme treatment indicate that the enzyme effected an opsonic action in that the trypsin treated organisms were taken up more rapidly and in larger numbers. The virulent treated cells did not have the "agents" required to repel a certain degree of contact with the phagocytic cells. This characteristic of the treated cells to be more readily "identified" could also be attributed to changes in the surface components of the bacilli. The possibility exists that the enzyme has much the same action on the tubercle cells as postulated for the red blood cell (31). Normal red blood cell surfaces are dominated by strongly acidic groups. Following trypsonization there would appear ionized groups of less acidic character, due perhaps to a breaking of the bonds during the loosening up process which occurs when trypsin acts on the protein surface of the red blood cell.

Similar investigations were reported by Dodd and Bass (41) with trypsin treated human red blood cells. Surface alterations and increased susceptibility to phagocytosis with rabbit macrophages were also indicated. Perhaps the same hypothesis for the in vivo removal and destruction of the enzyme treated tubercle bacilli can be postulated as for the removal of RBC or altered RBC. In both cases, whether the RBC are altered by trypsin in vitro or by disease physiology in vivo, or whether the tubercle cell is altered by trypsin in vitro or as a result of exposure to excessive
factors present in vivo, the altered surface changes are characteristic and both are more susceptible to phagocytosis. Evidence to supplement the above is shown by the large number of healthy polymorphonuclear cells present after trypsin has been instilled intrapleurally (34).

Conclusive evidence for the destruction of the treated bacilli within the macrophage in tissue culture, however, was not obtained. This does not mean that other physiological conditions along with the more rapid ingestion of these cells could not result in their total destruction. Two conditions, however, prerequisite for the possible destruction of the tubercle bacilli, i.e., the altered surface and the presence of more phagocytic cells, have been fulfilled.

Furthermore, the tubercle cell exposed by trypsin from its protective tubercle not only would exert an opsonic effect on the organisms but would also be exposed to specific antibodies. Additional therapy was encountered as a result of trypsin debridement by depriving the bacilli of the appropriate culture medium in the tissues for growth.

Thirdly, the virulence factor or factors may be related to the metabolism of the cells. Virulent organisms may be capable of using a cultural pabulum supplied by the host which avirulent bacilli do not possess.

To extend our problem in this direction, we had to assay in vitro the tryptogenic effect on these cells to determine in what manner the enzyme altered the cell and initi-
ated its destruction. The first step which had to be undertaken was to determine whether trypsin was inactivated by Dubos medium. This was determined by assaying aliquots of trypsin plus Dubos medium and trypsin alone. This was accomplished by using the assay method described previously. No inactivation of the trypsin was observed. Trypsin, therefore, was free to act upon the tubercle bacilli.

Various possibilities present themselves when analyzing the data. First of all, if the trypsin had stimulated instead of inhibited the organisms, as in the case of Micrococcus pyogenes var. aureus described by Grab (16), we would suspect hydrolysis of the proteins constituting the medium to have been degraded making them more readily available to the bacilli. Furthermore, an increase or decrease in the oxidation-reduction potential would exert a marked change on the growth pattern of these cells. However, since the effect with trypsin was inhibitory, and since the cells retained their cellular structure and at the same time lost the characteristic serpentine formation so characteristic of the virulent tubercle bacilli (16), it is felt that either the trypsin inactivated extracellular enzymes, released intracellular enzymes, or digested surface components responsible for this characteristic arrangement. Inactivation of extracellular enzymes could occur when the higher dilutions of trypsin were used. The enzyme inhibitors or certain repellent forces of the intact cell, in this
case, are totally inadequate. Furthermore, the enzyme may alter the cell wall not enough to cause lysis, but sufficiently to cause the entrance or exit of constituents inhibitory to growth. The inhibitory effect may also be a combination of both factors.

Because the trypsin treated cells showed the absence of the serpentine arrangement, failure to grow when subcultured, yet maintained their cell integrity, we proceeded further and investigated the changes in the metabolic activities of the cell which occurred after trypsin treatment. These alterations were produced by surface and/or permeability changes.

The metabolic characteristics of the virulent and avirulent strains may best be differentiated by comparing the two. First, the serpentine arrangement is characteristic of virulent cells in contrast to avirulent (16). Secondly, the saprophytic mycobacteria and certain avirulent variants of pathogenic strains, oxidize lactic acid more rapidly than do the virulent strains (17). Thirdly, the virulent bacillus has the capacity to utilize phospholipids, while the avirulent organisms have lost this capacity. Finally, differences may also be noted in the ability of the organism to grow in varying concentrations of oxygen and carbon dioxide (27).

In assessing the effects of surface and permeability changes caused by trypsin on the bacterial cells, it seems
unlikely that this enzyme enters the cell, but rather exerts its activity extracellularly. The surface alteration was definitely detected by the greater absorption of the dye on the surface of the trypsin treated cells in comparison with the absorption of the dye on the normal organisms. Layers of the cell were thus exposed by trypsin which had greater affinity for the dye, basic fuchsin (37), and perhaps different receptor groups for combination with dye. This phenomenon of surface alteration becomes more significant when we consider that we have already demonstrated a quantitative difference in the ingestion by macrophages of the treated and untreated bacilli. It is known, for example, that if the capsules of the pneumococci and streptococci are altered, the organisms become less resistant to phagocytosis. Although there exists at present no satisfactory explanation for this change, it appears that from the standpoint of the tubercle cell the same principle is involved, i.e., alteration of the surface components which rendered the organism more susceptible to ingestion.

Our next step was to determine if this alteration in the surface layer could result in a change in the metabolism of the tubercle bacillus. First of all, we had observed that the enzyme treated cells had lost the character-serpentine arrangement. This morphological characteristic was shown by Bloch to be due to a surface substance (cord factor) which causes the cells to arrange themselves in chains
and also side by side. We also removed substances from the outer layer responsible for this characteristic and in so doing altered the metabolism of the tubercle bacilli. This change was indicated by the fact that dehydrogenases are released which decolorized methylene blue. Whether these extracellular substances removed by trypsin digestion are similar to the polysaccharide cord factor of Bloch (8), which was extracted by petroleum ether, and whether these treated cells inhibit the migration of leucocytes (2), remains to be determined. In addition, Bloch observed a reduction in pathogenicity. Thus, the surface is altered to initiate more rapid ingestion and the metabolism is changed to produce possible destruction within the phagocytic cells.

Finally, we must not lose sight of the possibility of a specific acquired immunity after the sojourn of the organisms within the tissues for a long period of time. Furthermore, even in the presence of such acquired immunity, the defense mechanism involved in the formation of the tubercle may have altered the cell so that later after the tubercles are digested by trypsin the cells are more readily destroyed by the phagocytic cells. Such an immunity, however, resulted in destruction of the avirulent organisms but seemed to have only a restraining influence upon virulent bacilli.

With regard to the direct destruction of the organisms, it will be recalled that 500 mg was the highest concentration of trypsin tolerated intrapleurally (34). Thus, in
order to achieve direct killing of the tubercle bacilli, 5.0 mg/ml would be necessary. This concentration of trypsin would require instillation of 1000 mg trypsin in 200 ml of sterile physiological saline daily. Such a concentration would not be without harmful effects to the patient. Therefore, in contrast to the opinions advanced by others (5, 25), and since such a high concentration of trypsin was required to kill the organisms directly, the smaller concentrations of trypsin used by Reiser indicate additional factors must be present for the destruction of this organism in a tuberculous empyema. The range in our case between the 2.5 mg/ml amount which is bacteriostatic and the 5.0 mg/ml concentration which will kill the organisms is too high for therapeutic use.

The final demonstration of the reduced pathogenicity was obtained by infecting animals with trypsin treated cells and also initiating therapeutically intranasal inhalation of trypsin after the animals had been infected with Mycobacterium tuberculosis. An extended survival time was observed in all cases.

First of all, it is noteworthy to compare the guinea pigs infected with treated and non-treated cells and challenged with both types subcutaneously. When the non-treated tubercle infected guinea pigs were exposed subcutaneously to the trypsin treated cells, the characteristic immune response was observed. However, when the treated tubercle infected
guinea pigs were exposed to the non-treated cells, the reaction was quite different. In the latter case, a slight local lesion developed after injection which partially healed. In ten to fourteen days, however, a hard nodule appeared which broke open and remained as an ulcerating sore until the animal died. The regional lymphatic glands became swollen and underwent caseation. The subcutaneous injection of trypsin treated cells, on the other hand, resulted in the rapid localization of the area and no breakdown of the lesion. The entire area involved disappeared within sixteen days, and the lymphatic glands remained unaffected. A recovery of this nature indicated rapid removal of the treated cells by the phagocytic cells in the area. This phenomenon also indicated decreased resistance of the organism after trypsin treatment. It is felt, however, that if trypsin treatment is to be encouraged, this difference must be much greater than exhibited above and must result in the complete recovery of the infected animal. Furthermore, although our method of cell treatment and dispersion with trypsin proved to be the best for a large number of organisms, it is possible that some degree of clumping had occurred so that all the cells did not come in contact with the enzyme thus leaving some to proliferate later after injection.

Intranasal instillation of trypsin in tuberculous infected mice also showed an increase in survival time, but no survivors. In this case it is felt that better results
could have been obtained if more of the enzyme could have been instilled intravenously or intranasally via an aerosol powder. Trypsin therapy, however, altered the pathology of the lung, but not enough to completely inhibit tubercle formation. Nevertheless, it was shown that enzyme treatment reduced the pathogenicity of the organisms. Here again it is possible that not all of the trypsin solution given intranasally to mice was taken into the lung. This was due primarily to the narrow passages of the bronchioles and their tendency to constrict after inhalation of the first drop of the solution. The exact amount inhaled, therefore, was difficult to determine.
SUMMARY AND CONCLUSIONS

As a result of the experimental findings obtained in this study, the following conclusions can be formulated with respect to the action of trypsin upon *Mycobacterium tuberculosis*.

1. The tubercle bacilli do not undergo lysis when exposed to concentrations of trypsin ranging from 20 mg/ml to 5 mg/ml. Such concentrations, however, prevent further growth. Bacteriostasis was observed with 2.5 mg/ml. Further decreases in enzyme concentration failed to destroy the bacilli.

2. The enzyme treated mycobacteria retained cellular morphology and acid fastness, but lost the characteristic "serpentine" arrangement characteristic of virulent bacilli.

3. Utilizing aureomycin and dihydrostreptomycin potentiation of antibiotic activity with trypsin was demonstrated.

4. Quantitative differences in dye adsorption showed that surface modifications of the bacilli resulted from the exposure of the organism to trypsin. In addition, it was shown that trypsin treatment of *Mycobacterium tuberculosis* caused a characteristic permeability alteration. Release of intracellular enzymes (dehydrogenases), which decolorized methylene blue, and failure to grow when subcultured on Petragnani slants substantiated these findings.

5. Mycobacteria which had been exposed to trypsin were
-72-

more rapidly ingested, and in larger quantities, by rabbit macrophages in tissue culture. However, the macrophages could not more readily destroy the treated organisms.

6. An increase in survival time was demonstrated when trypsin treated mycobacteria were used to infect guinea pigs and mice. Furthermore, a prolonged survival time was again noted when trypsin was administered intranasally as a therapeutic agent to tuberculous infected animals. In addition, the trypsin treated cells which were introduced intradermally were localized and removed more rapidly from the injected area by tuberculous infected guinea pigs.
BIBLIOGRAPHY


I, Warren Eugene Engelhard, was born in Bowdle, South Dakota, October 24, 1922. I received my secondary school education in the public schools of Bowdle, South Dakota. My undergraduate training was divided among five schools: The University of Minnesota in Minneapolis, Minnesota; The Saint Thomas College in Saint Paul, Minnesota; The University of Louisville in Louisville, Kentucky; Columbia University of New York, New York; and Northern State Teachers College in Aberdeen, South Dakota. I received the degree Bachelor of Science in 1947 from the Northern State Teachers College, Aberdeen, South Dakota. In 1950 I received the degree Master of Science from the South Dakota University, Vermillion, South Dakota. In the autumn of 1950 I received an appointment as Graduate Assistant in the Department of Bacteriology at The Ohio State University. In the winter of 1952 I was awarded a fellowship sponsored by the Ohio Tuberculosis and Health Association at the same school. I held this appointment while completing the residence requirements for the degree Doctor of Philosophy.