A Thesis
on
Microbic Diseases
by
J. Lincoln Whitmire, M.D.
A

THESIS

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F. Lincoln Whitmore, M.D.
We are living in a century of progress. Every day civilization is advancing spiritually, morally, and intellectually. We are standing on the threshold of an era of absolute knowledge and knocking at the door-post of intellectual perfection. The time is coming when the unknown shall be known; when the mysterious shall be simplified; when the secrets of the grave shall be revealed and the mysteries of life and death shall be known to all.

In the past fifty years every branch of science has made rapid strides towards completion, and second to none are the sciences of Medicine and Pathology. The time was, in America, when to be a practicing physician it was necessary only to have a knowledge of the curative properties of a few dozen drugs, and a sufficient knowledge of the human anatomy to be able to bleed a patient, set a broken bone or reduce a luxation. All this is now past. A young physician on leaving medical college now is filled with facts and theories that his grandfather in the profession never dreamed of when he began; and the young man's pocket medicine-case is filled with drugs, the therapeutical action of which would have been to the old man, in his earlier days, as Greek and Latin to an American infant. Although there has been great and rapid advancement in the past fifty years, it is the result of the work of many ancestors.
centuries by chemists and surgeons who, alone in their laboratories, solved the mysteries that were to stand for a time between mankind and the grave; while the clergy and laity united in hindering, persecuting and abusing those who were working for their common good. But this spirit of animosity was influenced by the general advancement; and in its place came more liberal ideas as education became more general, so that today, instead of being antagonistic, Religion is in perfect harmony and accord with all the sciences and instead of hindering the other, they each lend a hand for their mutual advancement and improvement, while Concord leads them in their restless seeking after spiritual and intellectual perfection.

The advancement in Therapeutics has been more gradual, but no less important, than in other branches of science, while Pathology and Pathological Surgery has fallen into line with more rapidity, perhaps than any other branches of the science of Medicine and Surgery. This is due in great part to the microscope, if not entirely. To this little instrument we owe the glories of Pathology. It has opened up vast fields of information and for research, and it is the line and now of advancement in that line. Without it we could not be certain of the nature of any pathological formation, and without we have the connecting link between supposition and absolute certainty: between ignorance and knowledge. Among its many wonderful revelations
reveals in a great number of disease germs both of the Miasmatic and Zymotic varieties.

Before closing this article I will give the results of some personal investigations into
the general characteristics of one of the Zymotic variety.

The fact is generally conceded that all contagious diseases are germ diseases. They not
only originate from germs, but the infected tissues usually contain germs that are capable
of reproducing the disease. In most of these diseases the specific bacilli have been discovered,
isolated, cultivated and made to reproduce the disease, and many scientists go so far as to
say that all diseases are, and will some day be proven to be, of germinal origin.

In what manner do these germs enter the system? The human body is so constructed
that almost every portion of it is capable of absorbing foreign matter if that matter is in a proper
state to be absorbed. Along the whole alimentary canal are the principal absorbing glands. The
mucous membrane in any portion of the body is next in order. The serous membrane is
equally capable of absorbing, but owing to its position is less apt to be put to that use.
But the part that absorbs most readily is the subcutaneous tissue, and is therefore
best protected. A subcutaneous injection, an abrasion or a wound will allow it to
exercise its full absorbing power. By way of these avenues may a disease
germ enter the system. From the air we breathe, the water we drink, the food we eat, the articles we
Disease germs, like animals, thrive under circumstances peculiar to themselves. Some are more active in dry and others in moist atmosphere. Cold is fatal to some, while others can withstand a very low temperature. The same is observed in relation to heat. So with various antiseptic solutions used to destroy them. Some cannot live in the very weakest solution, while others seem to multiply in a moderately strong one. Boiling water will instantly destroy some, and others have to be boiled for hours at a very high temperature. It is our thorough knowledge of the chemical and physiological properties of these germs that makes us able to check the spreading of these diseases and to treat them when present.

It has been demonstrated that the direct cause of the constitutional symptoms present in many of these germ diseases is not the presence of the germ in the circulation, but is the presence third of the protamine, that the bacillus produces by its action on the protoplasm of the animal cells, which is absorbed. This product is the protamine of Delmi and is an alkaloid. It also produces certain derived albumins known as globulins and albumoses, to the absorption of which diseases owe their origin. To another class of animal alkaloids Santier has given the name of lucernamines, and he claims that they are products of tissue metamorphosis in the body. He is supported by Vaughan of Ann Arbor.
Breger, however, claims that they are really ptomaines formed in the intestines by the action of bacteria, and he is the highest authority on bacterial chemistry. In chemical and physiological action they are alike, and it is still disputed. A number of ptomaines and leucitomaines, as well as albumoses and enzymes are intensely poisons.

**Brief classification of Bacteria:**

<table>
<thead>
<tr>
<th>Type</th>
<th>Shape</th>
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</thead>
<tbody>
<tr>
<td>Ccci or Micrococi</td>
<td>Spherical</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>Clusters like grapes</td>
</tr>
<tr>
<td>Streptococci</td>
<td>Chain-like</td>
</tr>
<tr>
<td>Diplococci</td>
<td>In pairs</td>
</tr>
<tr>
<td>Tetrads</td>
<td>In 4's</td>
</tr>
<tr>
<td>Bacinae</td>
<td>In 4's-9's as cubes</td>
</tr>
<tr>
<td>Bacilli</td>
<td>Rod-shaped or oval</td>
</tr>
<tr>
<td>Spirilli</td>
<td>Cork-screw like</td>
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At one time the opponents to the germ theory of infection argued that the fact that certain symptoms followed the injection of pure cultures of bacteria did not prove that these results were due to the bacteria themselves, because, they claimed, other things besides the bacteria might be simultaneously introduced; which is true. Much of the controversy which has attended the study of bacteria has been due to this failure to differentiate the results.
results of the pneumoines, albumoines and enzymes of each specific bacillus. Take for example the unreliable results due to the injection of pure culture, which is apparent from the following list of factors in inoculation experiments, each of which produces different results.

In using infected tissue or excreta

1. The chemical constituents of the tissue itself.
2. The pneumoines.
3. Albumoines.
4. Leucomaines (if present)
5. Enzymes.
6. A combination of 1, 2, 3, 4, and 5.

In order to make inoculation experiments rigorously scientific the constituents of the culture must be used separately and it must be known what results follow the injection of
1. The pure culture itself; 2. The filtrate of the same; 3. Special alkaloid present; 4. Albumoines or globuloses; and 5. Enzymes.

Since Jenner discovered the protective power of vaccine against small-pox, all scientists have hoped that there would come a time when we would be able to inoculate against every contagious and infectious disease. The great difficulty which attends the prosecution of the
The subject of vaccination can be better understood by the enumeration of the conditions which modify the growth and products of bacteria, as follows: 1. Nature of the medium. 2. Effects of temperature (very striking). 3. State of the bacteria, i.e., parasitic or saprophytic. 4. Presence of other forms of bacteria, e.g., presence of blue pus in anthrax cultures. So that although we may pick out some certain microbe as the probable cause of the disease we are studying, there are so many hindrances in the processes immediately following that it is almost impossible to prove our claim; and when it comes to the final trial (reproducing the disease by inoculation) not the least hindrance is "The development of specific powers of resistance in the cells of the body to resist specific bacterial activities." A peculiar illustration of this is shown in the history of the discovery of preventative inoculation against anthrax, as follows.

Greenfield, June, 1850, discovered that the injection of the 12th generation of pure culture produced no result, and injections of earlier cultures in the series showed progressive loss of virulence. — Tousaint, July, 1850, discovered that the effect of the inoculation was modified by the temperature to which the body was raised that died of anthrax. — Pasteur, and later Lemoine, afterwards elaborated a system by which protective viruses may be prepared in large quantities. It was found that at a suitable temperature the attenuated
attenuated (shrunken) bacilli formed spores, and that these spores communicated their attenuated property to the bacilli cultivated from them. Thus it was easy to preserve the vaccinal material.

Kline discovered that the blood of a monkey that died of anthrax would protect sheep.

The first successful attempt to associate special bacteria with special fermentation processes or with special diseases was in the middle of the present century. Pasteur, 1857, discovered the ferments of wine and beer. Tollender, 1847, established the constant presence of rod-like bodies (anthrax bacilli) in cases of spleenic fever; and Koch, 1871, placed their association with that disease beyond a doubt as a primary cause.

Physicians have long been familiar with the fact that through the action of vegetable cells upon the protoplasm of the cell plants, certain basic substances are formed, known as alkaloids, which are alkaline in reaction, and which unite with acids to form salts. Again, when injected under the skin, or administered by mouth, in animals, these substances, alone or combined, produce energetic and specific effects upon the end organs of nerves, and upon the muscles themselves. Among this class are adropine, ephedrine, aconitine and strychnine.

Having gone briefly over the field of bacterial research, I will confine myself to one of the epidemic variety, the bacillus of diphtheria. Diphtheria is classed among the diseases of children, though one may contract it at any age. One attack does not protect the patient from
from another attack; on the contrary, once having it one is predisposed to other attacks. The name was first applied to the disease by M. Bretonneau of Tours. It is derived from the Greek word dipthera, a pellicle. It is an acute, specific, constitutional disease, both contagious and epidemic. Its usual place of appearance is in the throat, characterized by local exudation and glandular enlargements; attended by great febrile reaction and prostration of the vital forces and albuminuria. It has for its sequelae various paralyses, particularly of the muscles and nerves of the head and throat. — Cause — The cause of diphtheria is Klebs-Loeffler’s bacillus of diphtheria. The poison exists in the exudation and excretions of the fauces and in the breath, and floats in the atmosphere at a considerable distance from the original source. The bacillus, alighting upon the fauceal or other mucous surface, or on the skin (denuded of its epidermis), obtains there a nidus favorable for its development and propagation; but it does not enter into the interior of the system. It is not taken up by the lymphatics or blood vessels and conveyed to the internal organs. It remains localized upon the surface, and produces there the characteristic inflammation. Acting solely upon the superficial parts it cannot produce, in itself, systemic infection or blood poisoning, but as the poisonous snake or bee secretes a poison with contact of fangs or sting, it produces a poison called ptomaine, which is readily taken up by the lymphatics and blood vessels.
vessels and conveyed to every part of the system. So that the ptomaine, and not the bacillus, is the immediate cause of the systemic infection or poisoning from which so many victims of diphtheria perish. The period of incubation is from three to five days.

**Symptoms of Poisoning.** At first there is redness, associated with swelling, at the seat of infection. If it is in the throat the redness and swelling are associated with increased secretion of a mucous mucus. The redness spreads over the entire mucous surface of the mouth and posterior nares, when the exudation makes its appearance. This deposit may commence from one or more points. They gradually spread till the whole mucous membrane of the mouth and nose is covered. The color of the exudation may be white or slightly yellow, though it is usually gray. When black or dark brown it is from the presence of red blood corpuscles. On removing the membrane a raw and bleeding surface is exposed, slightly below the level of the surrounding mucous membrane, and at times ulcerated, which is speedily covered with a fresh deposit. The bleeding induration is a pathognomonic sign of diphtheria as differentiated from a erumpent exudation.

The lymphatic glands in the neck, that originate in the throat and fauces are swollen. The muscle tissue of the heart becomes soft and easily torn. Ulceration of the endocardium has been frequently observed. The kidneys undergo a granular degeneration in severe cases. The blood undergoes alteration, being black and fluid. In a mild invasion we have rigors
rigors succeeded by moderate fever, headache, languor, loss of appetite, stiffness of the neck, and tenderness at the angles of the jaw with slight soreness of the throat. In other cases the invasion is abrupt and severe. Chilliness, followed by great febrile reaction, 103°-105° F.

Pain in the ears, aching in the limbs, loss of strength, painful deglutition and swelling of the neck. Appetite poor, pulse at first full and strong, but it soon becomes frequent, slow but compressible. The urine is scanty, high-colored and contains albumen. The average duration is about nine days. This will give some idea of the signs and symptoms that follow the introduction of the protamines of diphtheria bacilli into the system. I will now give the results of some experiments made by me in the spring of 1889, while I was investigating the peculiarities and characteristics of Klebs-Löffler's bacillus of diphtheria.

At this time I was living in Chicago, attending the spring term of Rush Medical College especially for the course in Microscopy and Pathology. One morning the landlady told me I might change my room if I so desired as the doctor had told her that her little girl had diphtheria. Instead of leaving I availed myself of the opportunity this case offered to make some original investigations into the characteristics of the diphtheria bacillus. Supplying myself with the requisite apparatus (the different culture media I procured at the college—except the more simple ones, which I made myself) I proceeded as follows.
I took a straight stout platinum needle, sterilized it, and passed it into the membrane, rubbed it around so as to collect some of the exudation and withdraw it—using care not to touch it to any thing in withdrawing it. With this I impregnated my various tubes of culture media, and at the same time prepared some cover-slips. (See Ap. 40). After due preparation and time I possessed various colonies of bacteria.

I first used Loeffler's blood-serum mixture (See Ap. 41) of which I had five or six tubes. After twenty-four hours their surfaces were covered with thick irregular spots of a light-yellow color, slightly elevated above the surface in the center and thinner towards their irregular margins. A few deep orange-colored spots were scattered over the surfaces. My cover-slips, that were prepared when the culture media were inoculated, in almost each instance were found to show various organisms; but the most prominent among them were slightly curved bacilli of very irregular outline and size. In some cases they were clubbed at one or both ends, and a few were spindle-shaped. Some appeared as curved wedges, and some were irregularly segmented. Few if any were regular in outline. They were stained with the alkaline menthyline-blue solution of Loeffler (See Ap. 34. 35.) and many of the irregular rods were marked by circumscribed points in their protoplasm, which stained almost black. This irregularity in outline is the morphological characteristic of Loeffler's diphtheria bacillus.
Owing to the other organisms in the mouth (particularly of those who have decayed teeth) one cannot make a positive diagnosis of diphtheria from the exudate alone. The reason being that many of these organisms present the same characteristics as the bacillus of diphtheria when put to the microscopic test. The pathogenic activities of the diphtheria bacillus, together with its culture peculiarities when introduced into the tissues of susceptible animals, readily identify it. In kittens and guinea pigs the results of the growths are identical with those found in the bodies of human beings who died of diphtheria. The following is from my notes on its morphological and culture peculiarities.

It varies in size from 2.5 to 3.0 m long to 0.5 to 0.6 thick. Its shape varies even more than its dimensions. Some of which are: — Straight or slightly curved rods, with rounded ends. Often clubbed at one or both ends. Some forms stain uniformly, others irregularly. They usually appear as deeply stained granules in a lightly stained bacillus. In the blood-serum mixture, on which it grows best, and which is best adapted for determining its presence in diphtheria exudation, the colonies of the diphtheria bacillus grow so much more rapidly than other growths of organisms usually present in the exudation and excretions, that at the end of twenty-four hours they are often the only colonies present that attract attention, and if others of similar size are present, they are so different in appearance as not to be confused.
To be confounded with those of diphteria. Its colonies are large, round and elevated; grayish-white to yellow in color with the center more dense than the slightly irregular circumference. The surface is at first moist, and later dry, looking.

*Streptococcus Haar-agar* (See No. 6.) When this also the colonies present a characteristic appearance that differentiates them from others. They are more delicate in structure than upon blood-serum; they are more flat, dry, transparent, non-glistening and round-pointed and are not elevated above the surface of the medium. Slightly magnified, they appear granular and have irregular centers which are more dense than the surrounding zones, and darker by transmitted light. The margins are irregular and notched in appearance. They are always dry when deep down, coarsely granular and rarely exceed 3 mm. in diameter.

*Streptococcus* (See No. 12.) Here they develop slower than on other media that can be kept at a higher temperature. It usually takes seventy or eighty hours to develop fully, when they are in appearance flat, dry, translucent points, usually roundish in shape. Slightly magnified, the center is more dense than the surrounding zone. Sometimes there are two or more eccentric zones surrounding the center. The circumference is irregularly notched. Like those on agar-agar they are granular, and the deeper they are the more granular. In diameter they rarely exceed 1.5 mm.

Bonillon
Bouillou (see Ap. 13) "On this they usually grow in clumps, which generally fall to the bottom of the tube or become deposited on the sides. There are exceptions to this, however. Mine, unfortunately, were all exceptions (owing to carelessness in preparing the medium) and before I had a chance to prepare another tube the patches of inoculation had all disappeared from the throat of the patient.

Potato (see Ap. E.) On potato, at the temperature of 35-37°C (95-98°F), the growth for several days is invisible, only a thin dry glaze can be detected where the surface was inoculated. Microscopical examination, at the end of twenty-four hours, shows quite an increase in the number of organisms planted.

Slab and Slant Cultures. In slab and slant cultures, on both agar-agar and gelatin, the surface growth is seen to predominate over that along the track of the needle in the depth of the medium. Isolated colonies on the surface of either of the media in this method of cultivation present the same characteristics that have been given for the colonies. The bacilli in simple slab cultures do not extend laterally very far beyond the point to which the needle extended.

The diphtheria bacillus is non-motile. It does not form spores. It dies in ten minutes at 156°F. It grows at a temperature varying from 91.6°F to 99.6°F, but best at 98.6°F and in the presence of oxygen.

Staining. In cover-slip preparations, made either from the fauces or from 'pure' cultures,
it stains readily with ordinary aniline dyes. It also stains by Gram's method (see p. 12), but the best results are obtained from Loeffler's alkaline methylene-blue solution (see p. 7). This brings out the dark points in the protoplasmic body of the bacillus and aids thus in its identification.

Pathogenic Properties. When injected subcutaneously into the tissues of a susceptible animal (I used a kitten) the result, blood-poisoning, is not caused by the absorption of the bacilli into the circulation. They remain localized at the point of inoculation, never spreading beyond the nearest lymph glands. The ptomaines of the bacilli are however absorbed and produce the characteristic constitutional symptoms of diphtheria. A very small portion of fluid pure culture introduced subcutaneously at the root of the tail of one of my kittens produced death in three days. There was a great amount of inflammation at the point of inoculation, and the surrounding lymph glands were swollen. It experienced great difficulty in breathing and on dissection the pleura was found to be filled with serous fluid. The suprarenal capsules were slightly swollen. There was some dispute between my partner and myself whether there was fatty degeneration of the liver or not. A microscopical examination of the blood, the kidneys, the suprarenal capsules and the liver yielded negative results as to the presence of bacteria, and none were found in culture media inoculated from them.

With another kitten I experimented by opening the trachea and rubbing some pure culture
on the mucous membrane, which killed that kitten in five days. On dissection the same results were found as in kitten number one. All of which tends to show that the constitutional symptoms present in diphtheria are not caused by the absorption of the bacilli into the circulation, but by the absorption of the products of the bacilli.

Preparation of Nutrient Media. Appendix A. Blood-serum requires special care in its preparation. It is desirable under all circumstances to reduce the unavoidable contamination, which to a certain extent occurs during the manipulation, to its minimum degree. It is possible to collect serum from small animals and in small quantities under such precautions that it is perhaps not contaminated; but ordinarily for laboratory purposes a large quantity is needed, so that the slaughter-houses form the sources from which it is usually obtained, and here a certain amount of contamination is unavoidable. The animal from which the blood is to be collected should be drawn up to the ceiling by the hind legs, the head should be held well back, and with one pass of an aseptic and very sharp knife the throat should be cut clear through. The blood should be collected in jars prepared antiseptically. While standing to coagulate, a glass rod should be passed between the clots and the sides of the vessels to detach every part of the clot from them. Tightly cover and place in an ice-chest for forty-eight hours. The temperature should not
not be so low as to prevent coagulation. Draw off the serum with a sterilized pipette and place in tall cylinders which have previously been sterilized, and plug with cotton. Treat it all this way, avoiding the presence of blood coloring-matter, and put it in a cool place for another twenty-four hours; during which time the expensurant elements will sink to the bottom, leaving the supernatant fluid quite clear. This may then be pipetted off, either into sterilized flasks of about 100 c.c. capacity, or into small sterilized test-tubes of about 8 c.c. per tube. It is then to be sterilized by the intermittent method at a low temperature, viz. for one hour on each of five successive days at a temperature of 58° to 60° C. During the intervening days it is to be kept at the room temperature to permit of the development of spores, that may be present into their vegetative forms, in which condition they are killed by one hour's exposure to the temperature of 60° C. At the end of this time the serum in the tubes may be retained as fluid serum, or solidified at a temperature between 65° and 68° C. In solidifying the serum the tubes should be placed in an inclined position so that as great a surface as possible may be given to the serum. The process of solidification requires constant attention if good results are to be obtained, i.e. if a translucent solid medium is to result.

When solidification is complete the tubes are to be retained in the erect position and, unless intended for immediate use, must be prevented from drying. The superfluous cotton
cotton at the ends of the plugs should be burned off, and the mouth of each tube covered by a rubber cap.

Appendix 12. Bouillon. Five hundred grammes of finely chopped beef, free from fat and tendons, is to be soaked in one litre of water for twenty-four hours. During this time it is to remain in an ice-chest or to be kept in a very cold place. It is then to be strained through a coarse towel and pressed until a litre of the fluid is obtained. To this is to be added ten grammes (10%) of dried peptone and five grammes (0.5%) of common salt (NaCl). It is then to be rendered slightly alkaline with a saturated soda solution. The flask containing the mixture is then to be placed either in the steam sterilizer, in a water-bath or over a fire-flame; and kept at the boiling point till all the albumin is coagulated, and the fluid portion is of a clear, pale, straw-color. It is then filtered through a paper filter, and sterilized by the fractional method in a steam sterilizer.

Appendix 13. Gelatin. For this the bouillon is prepared as above except that the gelatin is added and thoroughly dissolved before the neutralizing material is added. The gelatin will dissolve very readily in hot bouillon. The reaction of gelatin as it comes from the factory is usually quite acid, so that a much larger amount of alkali is needed. The gelatin may be made to dissolve over the water-bath, in the steam sterilizer or over the free flame. If the flame is used then
thin watch carefully and stir constantly to avoid burning the mixture. When done, sterilize fifteen minutes a day for three successive days, then stop with cotton and put away.

Appendix C. Agar-agar. Prepare the bouillon as usual. Agar-agar reacts neutral, so that the bouillon may be neutralized before the agar-agar is added. Then add finely chopped agar-agar in the proportion to 1:1.5 to 1:5. Place in a porcelain-lined iron vessel and make a mark on the side of the vessel at which the level of the fluid stands after adding about 250 c.c. of water. Then allow the mass to slowly boil for three or four hours, being careful to maintain the original level of the mixture by adding water from time to time. When done, place in a cool pan of cold water and stir until it cools down to 60° C. Then you can add the whites of two eggs (which have been beaten up in 50 c.c. of water) without any danger of coagulating the albumen. Mix thoroughly through the agar-agar and allow it to stand for thirty or thirty-five minutes.

Appendix E. Glycerine is to be added if in done after filtration and before sterilization in proportion of 5:1.

Appendix E. Potato Medium. There are two ways. 1. The potatoes are taken as they come from market, old ones being preferred, and carefully scraped and scrubbed under the water tap until all the adherent dirt has been removed. The eyes and all discolored parts are
are then carefully removed. They are then placed in a solution of corrosive sublimate (1:1000) and allowed to remain twenty-four minutes; at the end of this time, without rinsing off the sublimate, they are placed in a covered bucket, with a perforated bottom, and sterilized for forty-five minutes. On the second and third days this is repeated for fifteen or twenty minutes. They must not be removed from the bucket till sterilization is complete. At the end of this time they are ready for use. Cut in halves and use the flat surfaces for inoculation. Everything that touches them from first to last must be aseptic.

II. In test-tube use one simply dibbles off the coarser particles of dirt with water and a brush, and with a cork-borer punches out cylindrical bits of potato that will fit closely into a test-tube. On each bit of potato is then to be cut a slanting surface running diagonally from about the junction of the first and second thirds of the cylinder to the diagonally opposite end. (See Fig.) These cylinders of potato are to be left in running water over night; otherwise they would be discolored by the process of sterilization. In the morning each half of the cylinder is placed in a tube with the slant surface up. Plug with cotton and sterilize with steam for forty minutes a day for three days. Then finished they appear as in figure 2. The slant surface receives the inoculation.
Appendix II. Loeffler's Alkaline Mentholine-Blue Solution. Be concentrated alcoholic solution of mentholine-blue, 30 c.c., caustic potash in 1:1000 solution, 100 c.c.

Appendix III. Cover-slip Preparation. All dirt and grease must be removed from the slide. To do this, immerse for a few hours in strong nitric acid - HNO₃, rinse in water, then in alcohol, then in ether and finally keep in alcohol to which some ammonium has been added. When ready to use, wipe dry with a clean cotton or silk cloth. Place in the center of one a small drop of distilled water or physiological salt solution. With a perfectly aseptic platinum needle take a small portion of the colony and mix carefully with the drop of water. Cover and evaporate carefully so as not to over heat. When dry, pass through the flame three times (bacteria side up), allowing one second for the passage through the flame. To stain the fixed preparation it is taken in the forceps and a few drops of a watery solution of coloring material is placed upon the evaporated surface, and allowed to remain for twenty or thirty seconds. The slip is then carefully rinsed in water, and without drying, is placed in the slide (bacteria side down). The excess of water is taken up by a blotter and it is ready for use.

Appendix IV. Gramme's Method. In this the objects to be stained are treated to an alkaline-water solution of gentian-violet made after the formula of Erlich, viz.
To 100 c.c. of water (distilled), aniline is added, drop by drop, and the solution shaken after each drop is added, till it has an opaque appearance. Filter through moist filter-paper till perfectly clear. To 100 c.c. of the clear filtrate add 10 c.c. of absolute alcohol and 1 c.c. of concentrated alcoholic solution of either fuschin, mentheline blue or gentian-violet. After remaining in this for about twenty or thirty minutes they are immersed in an iodine solution, viz. Iodine 1 gramme, iodide of potassium 2 grammes. Distilled water 300 c.c. In this they remain for five or six minutes. They are then transferred to alcohol and thoroughly rinsed. If still a violet color they are again treated to the iodine solution followed by alcohol. When the violet color vanishes they are ready for use.

Z. Lincoln Whitmire, M.D.
Urbana, Ill.