THE RELATIONSHIP OF SOIL PROTOZOA TO TUBERCLE BACILLI1.2

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The aim of the work outlined in this paper was to determine what rôle protozoa play in the destruction of tubercle bacilli in nature and it was carried out simultaneously with studies on the death rate of tubercle bacilli in soil and in association with various bacteria and fungi.

ISOLATING SOIL CILIATES

Preliminary studies on the problem dealing with the relation of protozoa to acid-fast bacteria, especially the tubercle bacilli, were largely concentrated on the relationship of a certain ciliate to several pure cultures of bacteria. This protozoan was found to be present in great abundance in a soil taken from a plot of the New Jersey Agricultural College and Experiment Station. The organism was found to develop readily on many bacteriological media tested. It has been identified as Colpoda Steinii.

As the relationship could not be determined with certainty in mixed cultures similar to the mixed population in which protozoa naturally live in soil, a method of purifying the cultures was undertaken. The procedure of repeated picking and washing the ciliate was similar to that employed by Peters (1921). A single protozoan was picked with a sterile micropipette. This was accomplished readily under a dissecting microscope. The protozoan was placed in sterile medium in a depression slide. After

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2 This work was carried out under the auspices of the Committee on the Microbiology of the Soil of the National Research Council.
about five minutes the operation was repeated and thus continued through seven sterile droplets. The protozoan was finally placed in the desired pure culture of bacteria and allowed to multiply. Control protozoa inoculated into nutrient broth and tested for purity were found to be free from bacteria. In the above manner the protozoan was successfully introduced into several pure cultures of bacteria in which it thrived. One of the bacteria was an unidentified small Gram-positive bacillus found to be predominating in a mixed infusion in which the protozoan grew excellently. The protozoan was later transferred to a culture of *Bacillus megatherium*, in which it grew profusely. It failed to thrive at a temperature of about 28° to 31°C. in an *A. aerogenes* culture although on some occasions the introduced cells multiplied readily. When the temperature was lowered to about 23° to 25°C., the protozoan was found to thrive with *A. aerogenes*. During the summer months placing the test tubes in a pan of water in a draft of air caused a lowering of temperature favorable to this ciliate.

There is no reasonable doubt as to the purity of the bacterial cultures in which the protozoan was induced to grow. Control transfers of the protozoa through sterile media constantly proved them to be free from bacteria, because of the failure to develop bacterial growth in nutrient broth or on nutrient agar. The protozoa could thus be freed of all the pure bacterial cultures in which they were grown. These bacteria would have quickly produced turbidity in nutrient broth. As an additional precaution to assure purity, the protozoan was transferred twice from each pure culture to another pure culture of the same bacterium, using the identical procedure employed in the original purification of the cultures. Culture studies did not show the presence of contaminating bacteria.

Three cells of protozoa freed from bacteria by transferring with a pipette through a sterile media were placed in each of three nutrient broth suspensions of Timothy 1589, a saprophytic acid-fast organism. The suspensions were observed for a period of one week. One and two days after the transfer many protozoa
were found to be present. Multiplication of the protozoan had actually occurred. The numbers were, however, rapidly reduced and after the seventh day the protozoa were no longer observed and could not be revived by transferring to fresh suspensions. Similar experiments with Avian 531 suspensions revealed that multiplication also occurred but even less readily than with the Timothy 1589 suspensions and no protozoa remained after three or four days. Bacteriological studies proved that no contamination had occurred during the manipulation. These experiments were conducted at summer room temperature which was later found to be too high for the best growth of the protozoan. Better success was obtained with the growth of the ciliate in Timothy 1589 and Avian 531 suspensions later, when a more convenient method of transferring the protozoan had been devised.

Because of the very tedious task of obtaining a sterile protozoan by the pipetting method and because only one cell could be introduced into a bacterial culture at a time, a more satisfactory method was sought. The protozoan encysted upon drying and the cysts possessed considerable resistance to heat and chemicals. The active protozoan in a liquid culture was destroyed by a temperature of 60°C in less than five minutes. A dried culture resisted 90°C for about twenty minutes and 85°C for over two hours. *Aerobacter aerogenes* was found to be rather easily destroyed after drying. The cultivation of the protozoan in a culture of *A. aerogenes* had already been accomplished. This pure culture of the protozoan and the bacterium was incubated at 23°C to 25°C. One drop of a four-day culture swarming with protozoa was added to a series of sterile test tubes. The moisture was allowed to evaporate in an incubator kept at 37°C. The dry tubes were heated in an oven to determine the temperature and time necessary to destroy the bacteria without destroying the protozoan cysts.

The medium used was found to alter greatly the thermal death point of the dry *A. aerogenes*. The bacteria in the drop of medium designated B resisted 95°C for one hour, while the bacteria in the dried drop from another medium, A, were almost always
destroyed by a temperature of 70°C. in one hour. The formulae for these media are:

"A" medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil extract</td>
<td>100.0 cc.</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.25 gm.</td>
</tr>
<tr>
<td>Water</td>
<td>900.0 cc.</td>
</tr>
</tbody>
</table>

"B" medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil extract</td>
<td>400.0 cc.</td>
</tr>
<tr>
<td>Peptone</td>
<td>1.0 gm.</td>
</tr>
<tr>
<td>Water</td>
<td>600.0 cc.</td>
</tr>
</tbody>
</table>

Soil extract

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil</td>
<td>1000.0 gm.</td>
</tr>
<tr>
<td>Water</td>
<td>1000.0 cc.</td>
</tr>
</tbody>
</table>

Boil ten minutes and filter

Bacteria become most numerous in the richer B medium. The difference in the thermal death point is probably influenced by the number of cells.

A dried drop of the four-day culture in the A medium heated at 70°C. for one hour proved to be usually bacteria free, while the protozoa remained viable. Cultures of the protozoan could be conveniently obtained by merely adding a pure culture of a bacterium to a test tube containing bacteria-free cysts. To determine the success of the method, pure cultures of the tiny Gram-positive bacillus were introduced into the test tubes containing the protozoan cysts. Active protozoa could be observed after one day. After several days, the protozoa swarmed through the media. Transfers from each tube to a d-glucose broth fermentation tube were then made. When A. aerogenes survived, gas formation was readily detected. The Gram-positive bacillus failed to produce gas in the d-glucose broth. Tests were developed for the detection of A. aerogenes in the presence of other bacteria and were used to check all the transfers. The organism, A. aerogenes, was not destroyed in about 15 per cent of the heated tubes.

When nutrient broth was introduced into a test tube containing the protozoan cysts, excystation was brought about. Active protozoa could be noted in one or two days. After about the
third day, no active protozoa were present. Bacteria-free broth failed to support growth of the protozoa.

Nutrient broth suspensions of Timothy 1589, Avian 531 and an acid-fast soil bacillus were placed in the test tubes containing the bacteria-free protozoan cysts. In the Timothy 1589 and Avian 531 cultures slow development of the ciliate occurred. The cultures were continued by making transfers to fresh suspensions of the bacteria. The ciliate failed to grow in suspensions of an acid-fast bacillus from soil; a few ciliates were still present on the fifth day; thereafter none were observed. No contamination of cultures occurred to detract from the accuracy of the observations. After two months cultivation, three cultures of the protozoan with the Avian 531 organism and three cultures of the protozoan with the Timothy 1589 organisms were still flourishing. Transfers to fresh suspensions were made weekly. Apparently the cultures could be maintained in this manner indefinitely. Colpoda Steinii has now been cultured with Avian 531 for more than one year. No foreign bacteria have entered the cultures. Transfers of the Avian 531 culture to nutrient broth did not become turbid nor did the protozoan live in this clear broth. If a contaminating organism capable of supporting the ciliate had been present, the ciliate would have continued to exist in the nutrient broth. No development of colonies occurred when the Avian 531 culture was plated on nutrient agar.

In later tests, Colpoda Steinii failed to develop in suspensions of M. ranae, a frog tubercle bacillus and in suspensions of eleven acid-fast bacteria of soil origin. One soil acid-fast bacillus furnished luxuriant growth of the protozoan. The H 37 human tubercle bacillus and the Ravenel bovine tubercle bacillus failed to support C. Steinii.

C. Steinii did not grow in the filtrate from a six weeks glycerol broth culture of Avian 531. The soluble constituents of the culture did not alone fulfill the food requirements of the protozoan.

CULTURAL RELATIONSHIPS OF ACID-FAST BACTERIA AND CILIATES

This group of experiments was devised for the purpose of determining the effect of protozoa upon acid-fast bacteria and
acid-fast bacteria upon protozoa. The main possibilities considered are:

1. The protozoa may be parasitized by the acid-fast bacteria and serve as carriers of disease, as many insects serve as carriers for mammalian parasites.

2. The acid-fast bacteria may be toxic to the protozoa and cause abnormalities in their functions and structures or retard normal development.

3. The protozoa may utilize the bacteria as food and ingest and destroy them.

A technic was devised for staining ciliates by the acid-fast method in order to determine whether ingestion of Mycobacteria occurred. The fixative found to be most successful was of the formula:

- Picric acid................................. 1.0 gm.
- Alcohol........................................... 200.0 cc.
- Formalin........................................ 40.0 cc.
- Glacial acetic acid.......................... 15.0 cc.

Equal parts of fixative and culture are mixed and allowed to stand at 37°C for thirty minutes. Excess fluid is removed by centrifugation and 0.05 per cent egg albumin added. The excess fluid is again removed and the material spread on a glass slide, allowed to dry slowly and stained by the Ziehl-Nielsen method.

Pure cultures consisting of *C. Steinii* and Avian 531 together and likewise *C. Steinii* with Timothy 1589 were thus stained. Many acid-fast bacteria could be seen within the protozoa. The number contained in some single protozoa was estimated at about 100. Large clumps of acid-fast material were dispersed throughout the ciliate and many intact bacilli were noted. Undoubtedly ingestion had occurred.

The problem was attacked upon the assumption that if multiplications of the acid-fast bacteria occurred within ciliates, the acid-fast bacteria would persist through repeated transfers of the protozoa to a fresh medium containing no acid-fast bacteria. Timothy 1589 is able to support the protozoa indefinitely when fresh transfers to nutrient broth are repeatedly made at intervals of about one week if surface growth of the bacterium occurs.
As the bacteria then develop outside of the protozoa this fails
to prove anything. Avian 531 fails to grow readily at the tem-
perature, about 23°C., favorable to the protozoa. A frog tuber-
cle bacillus was therefore procured from the Henry Phipps Insti-
tute at Philadelphia and used in the experiments. Six mixed
cultures consisting of known pure cultures were prepared. All
contained a tiny Gram-positive soil bacillus and in addition:

1. C. Steinii and Avian 531
2. C. Steinii and Timothy 1899
3. C. Steinii and M. ranae
4. N. cuculcus and Avian 531
5. C. cuculus and Timothy 1899
6. C. cuculus and M. ranae

The tiny Gram-positive bacillus served excellently to support
growth of the protozoa. The medium with which the cultures
were carried was such that the acid-fast bacteria were favored by
the presence of ammonia, phosphate and glycerol. Nutrient
broth furnished nutrients for sparse growth of the tiny Gram-
positive bacillus which could not use the glycerol. All the myco-
bacteria were capable of growing on the surface when carefully
planted, even with the Gram-positive bacteria contaminating the
liquid.

Medium "D"

Nutrient broth.................................................. 50.0 cc.
Water .......................................................... 950.0 cc.
Glycerol ....................................................... 10.0 cc.
Ammonium sulfate .............................. 1.0 gm.
Phosphate buffer ................................. 10.0 cc.

Phosphate Buffer

K2HPO4 ........................................................... 60.0 gm.
KH2PO4 .......................................................... 20.0 gm.
Water .................................................. 1000.0 cc.

After twenty-four hours, stains revealed that acid-fast bac-
teria had been ingested by the protozoa of all cultures. The
presence of acid-fast bacteria did not depress the numbers of
protozoa nor cause observable damage or stimulation to them.
The next day all cultures were transferred, 1 cc. of culture being
added to 10 cc. of sterile medium. After five days, examination revealed that some, but only a very few, acid-fast bacteria were contained in the protozoa. Fresh transfers were made as before. After five days examination was made again. No acid-fast bacteria could be found. This indicated that multiplication of acid-fast bacteria did not occur within ciliates, even when the medium in which the ciliates grew was very favorable for acid-fast strains.

Jordan (1932) detected no evidence that tubercle bacilli multiplied within Colpidium campylum.

To determine the effect of ciliates on the acid-fast bacterial numbers, pure cultures of the following composition were prepared:

1. Gram-positive bacillus and *Avian 531*
2. Gram-positive bacillus *C. Steinii* and *Avian 531*
3. Gram-positive bacillus *C. Steinii* and *M. ranae*
4. Gram-positive bacillus *C. cuculus* and *Avian 531*
5. Gram-positive bacillus *C. cuculus* and *M. ranae*
6. Gram-positive bacillus and *C. cuculus*

Medium *D* was used because it favored acid-fast bacteria and because the protozoa developed profusely in it when the tiny Gram-positive bacillus was included. A sufficient population of protozoa could not be obtained when the tiny Gram-positive bacillus was omitted. Of the medium, 5 cc. amounts were inoculated with 1 cc. of desired culture without the acid-fast bacteria and allowed to incubate for three days at 23°C. To 5 cc. amounts of sterile medium, 1 cc. transfers were again made and allowed again to incubate for three days so that the protozoa would be numerous. One cubic centimeter of a *D* medium, acid-fast suspension was added to each culture as desired; the mycobacteria had been dispersed by shaking with glass beads.

Twenty-four hours later all bacteria were counted by a special technic. An aliquot was taken and the Gram-positive bacilli counted by ordinary plating. Then to each suspension was added 5 cc. of 2 per cent sodium hydroxide. Five minutes action was permitted and the material was plated on crystal-violet-glycerol-agar. On these plates only acid-fast bacteria developed. *M. ranae* failed to form colonies so no counts were obtained for it.
Each culture was duplicated. The counts are:

<table>
<thead>
<tr>
<th>Group</th>
<th>Protozoa</th>
<th>Gram-positive bacilli</th>
<th>Avian 531</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (No protozoa)</td>
<td>0/cc.</td>
<td>520,000,000/cc.</td>
<td>84,000/cc.</td>
</tr>
<tr>
<td>2. (With C. Steinit)</td>
<td>2,000</td>
<td>20,000,000</td>
<td>40,400</td>
</tr>
<tr>
<td>4. (With C. cuculus)</td>
<td>1,000</td>
<td>39,000,000</td>
<td>9,200</td>
</tr>
</tbody>
</table>

The above technic was repeated to check results. Counts were made 12 hours and 60 hours after the mycobacteria were added. The Avian 531 counts are given below.

<table>
<thead>
<tr>
<th>Time after Avian 531 was added</th>
<th>Culture with C. Steinii</th>
<th>Culture without C. Steinii</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 hours</td>
<td>162,000</td>
<td>660,000</td>
</tr>
<tr>
<td>60 hours</td>
<td>1,640,000</td>
<td>1,600,000</td>
</tr>
</tbody>
</table>

An additional experiment conducted after experimental methods of counting Avian 531 were more satisfactorily worked out revealed that Avian 531 numbers changed from 15,000,000 to 25,000,000 per cubic centimeter in C. Steinii and in C. cuculus cultures in two weeks. The plate count was the same for the culture without protozoa. The same Gram-positive bacillus used in the previous experiments was included. Each count was based on the average of 6 plates, and each count was duplicated. It was thus demonstrated with certainty that the protozoa did not have a pronounced destructive action on this tubercle bacillus. The slight observed increase in Avian 531 is not of significant magnitude to indicate an actual increase in Avian 531 numbers. There is generally an increase in the plate count of Avian 531 following dispersion in a liquid medium, conditions being similar to those of this experiment.

There was considerable evidence that an excessive concentration of tubercle bacilli in a medium was unfavorable to protozoa. When a very concentrated Avian 531 suspension was added to a culture of protozoa in a bacterial medium well suited for rapid development of the protozoa, the protozoa were decidedly inhibited as evidenced by population. Thus, Avian 531 added in excessive quantities to vigorous C. Steinii and C. cuculus cultures on A. aerogenes or other bacteria diminished the multiplication rate of these protozoa. This is easily explained, for Avian 531 is a poor food for C. Steinii as compared to many other bacteria.
When the protozoa are induced to ingest vast quantities of the poor food, the growth rate is diminished. Also, even a high concentration of bacteria better suited as protozoa food may check growth.

**MORPHOLOGIC STUDIES OF THE ACID-FAST BACTERIA DURING INGESTION BY CILIATES**

With due consideration for the prevalent knowledge of cellular digestion, it was deemed likely that marked changes in the morphology of the acid-fast bacteria would occur as a result of ingestion of ciliates. The ingested bacilli could be readily studied when stained by the acid-fast procedure. An extended study of the bacilli was therefore made.

The bacilli occurring within *C. Steinii* grown upon pure cultures of Timothy 1589 and Avian 531 were first observed. Large masses of acid-fast bacteria were found in which the individual organisms were often indistinguishable. This did not necessarily indicate a homogenization of the bacilli to a mass of acid-fast material, however, because whenever fewer bacilli occurred in a ciliate, the bacilli appeared to be unchanged from those of the original culture. The masses probably tended to appear homogenous because of the compact packing of the individual bacilli and because the decolorizer failed to remove the dye completely from the central portion of the ciliates. The tendency of dye retention was frequently noted.

Many observations of acid-fast bacteria were made at various time-intervals following the addition of mycobacteria to an active pure mixed culture of a ciliate and the tiny soil bacillus. The amount of mycobacteria suspension was so regulated that large masses did not accumulate, the individual bacilli thus being distinguishable within the protozoa. Some ingestion occurred immediately after addition of the acid-fast bacteria. During a week of observations of Avian 531 cultured with *C. cuculus* and *C. Steinii* no morphologic changes were noted as a result of ingestion. Observations were rendered somewhat difficult by the pleomorphism of Avian 531. The bacilli varied in size and shape and in degree of granulation. No morphological change has been attributed to ingestion of mycobacteria by *C. cuculus* or *C. Steinii*. 
Isolation of pure cultures of a soil amoeba

Soil amoebae were isolated for the purpose of studying the relation of this group of organisms to the acid-fast bacteria. The isolation procedure is essentially the same as described by Severtzoff (1921).

A particle of soil was placed on the center of a cross produced on an agar plate by streaking a pure culture of *Aerobacter aerogenes*. The agar medium consisted of 100 cc. of nutrient broth, 900 cc. of water and 15 grams of agar. Less agar was used by Severtzoff, but the amount of exuded water caused the undesirable bacteria to spread too rapidly to the ends of the crosses. The plates were kept under bell jars at about 25°C. An amoeba was soon noted on the plates. After about a week transfers were made from the outermost portions of the crosses to the center of other similar plates. Transfers were repeatedly made in this manner. Microscopic examination of the plates revealed the progress of the amoeba along the streaks. Transfers were always made as soon as they reached the tip of the cross. A single amoeba was picked on one occasion so that the strain would be pure. After seven or eight transfers the amoeba was found to be in a pure culture of *A. aerogenes* as far as bacteriological studies could be used to determine the purity. Stained smears and colonies were typical of the pure culture. None of the bacteria survived toluol treatment for more than fifteen minutes.

It was necessary to free amoebae from the *A. aerogenes* in order that the amoebae could be introduced into pure cultures of acid-fast bacteria. Toluol treatment of the mixed culture was tried. The amoeba survived only for six minutes so this method could not be considered successful. Although other means could have been tried it was deemed best to seek a more resistant type of soil amoebae. Normal soil was treated with toluol for three hours and used for the purpose of isolation. It was believed that only protozoa which form very resistant cysts would survive. Many amoebae survived and two distinct types were induced to live upon pure cultures of *A. aerogenes*. Thirty minutes treatment with toluol destroyed the *A. aerogenes* and left the amoeba cysts viable. These cysts could then be transferred, free from
all contaminants, to any bacteriological medium or culture desired.

Amoebae and mycobacteria

Amoebae and acid-fast bacteria were introduced on the same agar plates to study the effect of the acid-fast bacteria upon the protozoa. All the culturing was done on an agar medium containing 100 cc. of nutrient broth and 15 grams of agar per liter. The amoebae were carried with pure cultures of *A. aerogenes*. The amoebae were added to all plates in aqueous suspensions taken from slant cultures. Acid-fast bacteria were added by means of a finely dispersed, filtered, aqueous suspension. Two distinctly different types of soil amoebae and three mycobacteria, Avian 531, *M. ranae*, and Timothy 1589 were used.

At first the acid-fast bacteria were added to the plates at the time the amoeba *A. aerogenes* suspensions were added. It was noted that amoebae were much retarded by the mycobacteria. No desirable active cultures were procured. Amoebae and acid-fast bacteria were successfully grown together when the amoebae were permitted to develop with *A. aerogenes* on an agar plate and the mycobacteria added after this development had occurred.

Preparations for staining were then made. The amoebae were suspended in 5 per cent acetic acid containing 0.1 per cent egg albumin by allowing the solution to wash about the surface of the plate. This material was placed on a glass slide and permitted to dry slowly. The Ziehl-Nielsen technic of staining was used. The conclusions drawn from the observations are:

1. Mycobacteria suspensions added to plates of agar medium hindered the development of amoebae.
2. Acid-fast bacteria placed on agar plates with amoeba soon collect about or within the amoeba cells.
3. Encystment is hastened by the mycobacteria.
4. No fat globules were noted in the amoebae with or without acid-fast bacteria, Sudan III being used as the fat stain; therefore, no abnormal fat distribution was observed.
5. The mycobacteria may have hindered the amoebae in several ways. *(a)* By toxic products. *(b)* By acting as indigestible
SOIL PROTOZOA AND TUBERCLE BACILLI

bodies which prevent normal feeding. (c) By a combination of factors.

No very good evidence can be here presented for any of these views. The acid-fast bacteria were not observably disintegrated by the amoebae. Therefore toxic products are somewhat difficult to account for. The great number of mycobacteria grouped about some amoebae indicated that these amoebae might have collected sufficient indigestible material to hamper normal digestion processes.

SUMMARY

1. Several methods were devised for conveniently and rapidly introducing pure cultures of protozoa, both ciliates and amoebae, into any pure culture of bacteria desired.

2. A culture of Colpoda Steinii was introduced into a nutrient broth suspension of Avian 531. The ciliate multiplied and was cultured thus for more than one year. Nutrient broth alone or the filtrate of a glycerol broth culture of Avian 531 did not support growth of the ciliate.

3. Colpoda Steinii and Colpoda cuculus do not destroy Avian 531, as evidenced by plate count studies.

4. Some acid-fast bacteria support growth of C. Steinii, others do not, a high degree of specificity being manifested.

5. Stains revealed large numbers of acid-fast bacteria to be ingested by C. Steinii and C. cuculus.

6. Several strains of soil amoebae failed to grow upon pure cultures of acid-fast bacteria.

7. Excessive concentrations of acid-fast bacteria inhibit protozoa.

REFERENCES