Resumen por el autor, Ivan E. Wallin.

Sobre la naturaleza de las mitocondrias.

I. Observaciones sobre los métodos de teñido de las mitocondrias aplicados a las bacterias.

El autor ha teñido bacterias mediante los métodos más empleados para el teñido de las mitocondrias, especialmente el verde janus. Todos los métodos procados tiñen las bacterias. Los métodos para el teñido de las mitocondrias, incluso el de coloración vital mediante el verde janus, no son específicos para las mitocondrias sino que tiñen también bien las bacterias.

II. Reacciones de las bacterias a los tratamientos químicos.

El objeto de estos experimentos ha sido buscar una diferencia fundamental en el comportamiento de las bacterias y las mitocondrias bajo la acción de ciertos agentes químicos empleados para determinar la naturaleza química de las mitocondrias. El autor no ha encontrado diferencia fundamental alguna en estas reacciones.

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ON THE NATURE OF MITOCHONDRIA

I. OBSERVATIONS ON MITOCHONDRIA STAINING METHODS APPLIED TO BACTERIA

II. REACTIONS OF BACTERIA TO CHEMICAL TREATMENT

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ONE PLATE (NINE FIGURES)

INTRODUCTION

The publication of Altmann's 'Bioblast theory' ('90) stimulated a new interest in the investigation of cytoplasm. The minute bodies observed by Altmann in the cytoplasm were thought by him to be the ultimate units of life, and the cytoplasm itself was considered a more or less passive and lifeless substance. This conception of cytoplasm and the contained bodies or granules has received no support from recent investigators. The bodies in question have come to be considered normal cytoplasmic organs by most investigators. They have been described by a great number of authors under various names. More recently the term 'mitochondria,' first used by Benda ('98), has come into general usage.

Following the pioneer work of Flemming ('82), Altmann ('90), and Benda ('98), a massive literature on mitochondria has accumulated. This literature has dealt chiefly with the presence or absence of mitochondria in the various types of cells in both plants and animals. Cowdry ('18) has given an exhaustive review of mitochondrial literature and has summed up the total of our knowledge of mitochondria.

It is quite apparent, from a perusal of Cowdry's excellent review, that we have an exceedingly limited knowledge concern-

ing the fundamental properties of mitochondria. Attempts have been made to investigate their physiological properties, but aside from a possible relationship to chloroplast formation in plants, nothing definite, apparently, has been established concerning their function. Regarding the chemistry of mitochondria investigators generally agree that they are of the nature of phospholipins and lipoids and perhaps contain some albumin. The theory of their chemical nature is based on their reactions to staining methods and various chemicals. 'Artificial mitochondria' were produced by Löwschen ('13) by the use of lecithin in different salt and albumin solutions.

Considerable study has been given to the morphology of mitochondria. The result of this type of work has led to the conclusion by Cowdry ('18) and others that the form of mitochondria is variable and after all of little importance. Two forms of mitochondria predominate, namely, rod-shaped and globular forms. Besides these two predominating types various irregular forms may be found.

An important consideration in the demonstration of mitochondria is the technique. This technique warns to the exclusion, in the chemicals employed, of various solvents of mitochondria, chief of which are ether, alcohol, and acetic acid. It has not been claimed for the majority of mitochondria staining methods that they are specific for mitochondria. This 'specificity,' apparently, has reference to other materials in the cell. However, it must be assumed that these methods, if they are to be of value, must have a relative specificity for mitochondria. The janus green vital staining method has been definitely placed in a class of specific stains for mitochondria by Cowdry ('18, p. 43).

The striking resemblance of mitochondria to bacteria is apparent to all who are familiar with the two groups of structures. This resemblance has been noted by various authors and has led Cowdry to suggest a division of mitochondrial literature into two periods: an older literature in which mitochondria were observed in cells and mistaken for bacteria and a newer literature in which they have been observed and recorded under various names.

The chief methods employed in cytological studies are based on the reactions of stains and chemicals on protoplasm. In many cases a differentiation between cells and cell structures is demonstrated solely by staining reactions. While such methods may be criticized on account of the absence of a definitely indicated specificity, their value cannot be denied, especially in cases where the difference is pronounced. It is fair to demand when structures bear so close a resemblance to each other as mitochondria do to bacteria that some method must be employed that will differentiate between the two if they are to be considered distinct structures.

Cowdry ('18 p. 72) says: "It occasionally happens that tissues prepared for mitochondria have been invaded by bacteria, in which case the bacteria stain just like the mitochondria by the Benda method, with iron hematoxylin and with fuchsin methyl green. I have found that large bacilli contain granules which stain intensely and apparently specifically with janus green. They resemble in distribution the so-called polar granules. Smaller forms often stain diffusely." It is not clear from this statement whether Cowdry means to limit this staining reaction of bacteria to those forms that have invaded cells or if he implies that bacterial smears fixed and stained by mitochondrial methods will give the same results. In another place, Cowdry ('18, p. 135), referring to mitochondria, says: "Fortunately, *they may be easily distinguished from bacteria by their staining reactions* (particularly to janus green), by their occurrence in almost all cells, by their behavior and by their lack of independent motility."

This latter statement would appear to imply that all bacteria possess independent motility. This would be contrary to established fact in bacteriology. Just what 'behavior' of bacteria is specifically characteristic is not indicated by Cowdry.

Concerning the staining reaction of bacteria to janus green, I cannot agree with Cowdry that "mitochondria are easily distinguished from bacteria" by this staining method.

The practically universal occurrence of mitochondria in plant and animal cells points to a fundamental property of these struc-

tures. Their nature remains as much a puzzle to-day as when they were first discovered. It is with a desire to point out certain similarities between mitochondria and bacteria besides the similarity of form as well as seek a specific differentiation between the two structures that these studies have been undertaken.

MATERIAL AND METHODS

The materials used in this investigation have included a large number of strains of bacteria, some from known pure cultures and others from various mixed infections. The mixed specimens were obtained from sputum from hospital patients, pus centrifuged from urine, pus from a carbuncle, cultures made from the intestinal contents of rabbits and kittens, cultures made from lymph nodes, and from various other sources.

The staining methods employed were: Bensley's acid fuchsin methyl green method, Schridde's modification of Altmann's method, Benda's crystal violet method, the copper hematoxylin method and the vital janus green method.

In the second part of this study a number of strains of bacteria were subjected to the action of alcohol, ether, chloroform, acetic acid, formaldehyde, potassium bichromate, osmic acid, and heat. The object of these experiments was not to determine the exact nature of the response of the organisms to these chemicals and heat, but to determine the effect on the staining reaction of the bacteria after such treatment. In every case controls were stained with the same stain used on the experimental preparations.

The janus green used in the vital staining was one of two lots that were kindly donated to the author by Professors Bensley and E. V. Cowdry. This opportunity is taken to express appreciation for this helpful courtesy. Viable cultures of human and bovine tubercle bacilli were supplied by Dr. Harry Gauss, of the National Jewish Tuberculosis Sanitarium in Denver.

I am especially indebted to my colleague Dr. Severance Burrage, of the Department of Pathology, for valuable assistance and suggestions in this work and also for generous use of bacterial cultures in his laboratory.

I. OBSERVATIONS ON MITOCHONDRIA STAINING METHODS APPLIED TO BACTERIA

In the following staining methods in which a fixation preceded the staining, smears were made in the usual way on the slide. Before the smears had time to dry they were immersed in the fixatives of the different methods and later treated according to the procedure for the particular method. In a few instances bacteria were centrifuged, fixed en masse, embedded, and sectioned.

The procedure in the janus green vital staining followed the method used by Cowdry ('14) for blood cells.

a. Bensley's acid fuchsin methyl green method

This method was used according to the directions given by Bensley ('11). It was found that the time for both fixation and staining could be shortened considerably with excellent results, obviously due to the more rapid penetration in the bacterial smears. In a number of instances the method was altered with a modified Flemming's fixation. This modified fixative consisted of osmic and chromic acids in the following proportions: 4 cc. 2 per cent aqueous solution of osmic acid and 6 cc. 1 per cent aqueous solution of chromic acid. This modification appeared to give a more rapid fixation and also good staining results with bacterial smears.

Besides a large number of unknown bacteria, the following strains were subjected to this method: human and bovine tubercle bacilli, Bacillus coli communis, Bacillus bulgaricus, Bacillus megatherium, Bacillus subtilis, Staphylococcus pyogenes aureus, Staphylococcus albus, and a pneumococcus.

In every case where this method was used the bacteria were well stained. In the majority of cases they were sharply stained.

b. Schridde's modification of Altmann's method

This method was used only to the extent of determining a positive staining in a few cases. The same difficulty experienced in demonstrating mitochondria with this method was experienced

with bacteria. Bacillus bulgaricus, Bacillus coli, and Staphylococcus pyogenes aureus were definitely stained by this method.

c. Benda's crystal violet method

A fairly large number of known and unknown bacteria were subjected to this method. Bacteria responded to this method just as mitochondria do. It gave the sharpest differentiation of bacteria obtained in any case where mitochondrial methods were used. Compared with Gram's stain, for example, on sputum smears, it gave a sharper differentiation. Here, also, it was found that the time for fixation and mordanting may be reduced considerably.

d. Copper hematoxylin method

This method was applied to only a few strains of bacteria. In some cases the staining of the bacteria was quite faint. This was particularly true after fixation with Zenker and the formalin-Mueller used with the Altmann-Schridde method. After Bensley's and the modified Flemming fixations the bacteria were stained very sharply by the copper hematoxylin method.

e. Janus green vital staining method

This method was used as prescribed by Cowdry ('14) in a 1:10,000 dilution in physiological salt solution. The dye was first tested by applying it to lymphocytes from a lymph node of the rabbit. It was found to stain the mitochondria of the lymphocytes as described by Cowdry.

The following results will serve to indicate the staining reaction on bacteria:

1. Human bacillus tuberculosis, viable strain. The bacilli stained rather faintly, the granular forms were easily recognized on account of the more intense staining of the granules. Observed ten hours after the preparation was made, the bacilli appeared to be stained slightly deeper.

2. Bovine bacillus tuberculosis, viable strain. The bacilli stained perhaps a little fainter than the human strain. Observed

three hours after the preparation was made, the bacilli did not appear to have absorbed any more of the dye.

3. Bacillus subtilis. A few moments after the preparations were made, deeply stained granules could be observed in the bacilli, while the cytoplasm of the bacilli was very faintly stained. In some bacilli the granules were very small, in others they were quite large. Figures 1 to 3 are camera-lucida drawings of some bacilli from these preparations after different lengths of time in staining.

4. Bacillus megatherium. The preparations contained a great number of spores besides the bacilli. The spores appeared to be tinted by the dye. The staining reaction of the bacilli varied in different preparations, apparently depending upon the age of the culture. In some cases the cytoplasm was distinctly stained, while in other cases it was not stained, but contained intensely stained granules. Figures 4 to 6 represent camera-lucida drawings of bacilli from various cultures with different lengths of staining time. In one preparation the cytoplasm was quite intensely stained immediately after application of the dye. When it was examined three hours later, the majority of the bacilli had swelled to about three times the normal size and contained very large intensely stained granules. A drawing was not made of this preparation and I have been unable to get the same results again.

5. Unknown bacilli and cocci from a mixed culture. Both the bacilli and cocci were intensely stained immediately after preparation was made. There were a number of bacilli that were unstained. Obviously, it could not be determined in the preparation if they belonged to the same strain that did absorb the dye. Observed ten hours after the preparations were made, a number of the bacilli were swollen and contained large intensely stained granules, other bacilli were unstained.

6. Unknown bacilli and spores, apparently a pure culture, made from the intestinal contents of a rabbit. The bacilli were intensely stained immediately after the dye was applied. The spores appeared to be tinted by the dye.

7. Unknown bacilli and spores, apparently a pure culture made from the intestinal contents of a five-day-old kitten. The bacilli were moderately stained, no granules apparent. The spores did not appear to have absorbed any of the dye.

8. Unknown cocci, culture made from a human throat swab. The cocci were moderately stained, no granules apparent.

9. Unknown bacilli, culture made from a lymph node of a rabbit. Apparently not a pure culture. Some bacilli stained faintly, others quite intensely. Some large bacilli that were faintly stained contained intensely stained granules.

10. Unknown bacilli and spores, culture made from a lymph node of a rabbit. The bacilli were moderately stained. The spores were decidedly tinted by the dye.

11. Unknown bacilli and cocci, culture made from a lymph node of a rabbit. Bacilli and cocci were moderately stained.

12. Unknown cocci, culture made from a lymph node of a rabbit. Preparation contained cocci of two sizes. Larger cocci were intensely stained, the smaller forms were moderately stained. The difference in staining was also demonstrated in the two forms when they were stained with Loeffler's methylene blue.

13. Bacillus coli, pure laboratory culture. The bacilli stained intensely immediately after the dye was applied, a few forms were only faintly stained. After the stain had acted for five and a half hours, the majority of the bacilli were swollen and contained a single large intensely stained granule. Figures 8 to 9 are camera lucida drawings of the preparation immediately after it was made and five and a half hours later.

DISCUSSION

The results recorded above demonstrate that the mitochondrial methods used are not specific for mitochondria, but that they also stain bacteria. The intensity of the stain varied with the different strains of bacteria used and apparently there was a variation in intensity with the different methods on the same strain of bacteria. Such variations apparently, also occur with mitochondria. The janus green vital staining method appeared to be the most delicate of the methods used.

The effect of janus green on tubercle bacilli was contrary to expectation. On account of the fatty envelope of these forms, it was to be expected that they might stain more intensely than any other bacteria. This would imply that fats, waxes, and lipoids should respond in a like manner to a given stain. Such an inference may not necessarily be true. However, the proof that mitochondria are of a lipoidal nature is far from conclusive. While there is nothing specially indicated as to the chemical nature of the bacteria that were stained by janus green, it would appear that one is justified in concluding that these bacteria and mitochondria do have something in their chemical structure that is common to all.

The different reactions of a strain of bacteria at different periods in the life of the culture to janus green is suggestive. It would appear that janus green has possibilities as a delicate indicator of the physiological state of certain strains of bacteria.

II. REACTIONS OF BACTERIA TO CHEMICAL TREATMENT

The behavior of mitochondria when subjected to various chemicals and heat has been one of the chief methods used in determining the nature of these bodies. N. H. Cowdry ('17) made a detailed study of the comparison of mitochondria in plant and animal cells. The behavior of the two groups of mitochondria under the influence of various chemicals (ether, alcohol, formaldehyde and acetic acid) as well as their morphology was the method employed in this comparative study. Cowdry concludes that there is no difference between the mitochondria of plants and animals.

It must be admitted at the outset that in most instances there is nothing specifically indicated in the reaction of minute microscopic particles to chemicals. With perhaps a few exceptions, these reactions are only relative. For example, ether acting upon tubercle bacilli for a limited time will extract a fat (supposedly forming an envelope for the bacillus) from the organism. From such a reaction there is nothing indicated as to the particular kind of fat that has been dissolved. However, inasmuch as these

methods have been used not only in comparing the mitochondria of plants and animals, but also in determining the approximate chemical nature of mitochondria, it is necessary in this comparative study of bacteria and mitochondria to also determine the reaction of bacteria to these chemicals. It must also be admitted that there is no basis for supposing that all strains of bacteria should respond in the same way to a given chemical. It has been indicated by Cowdry and others that all mitochondria do not respond to a given chemical in the same way.

The methods employed in this study of the reactions of bacteria to chemicals were designed to retain as much as possible of the materials resulting from the chemical action. Metal rings coated with paraffin were sealed to microscopic slides, smears of the bacteria were then made inside of the rings, and after the chemicals were added cover-glasses were sealed over the rings to prevent evaporation. After a given time the cover-glasses were removed and the chemical was permitted to evaporate. When the smears had thoroughly dried and the paraffin around the smears had been removed with xylol, a thin film of celloidin was painted over the smear. The smears were then stained, using the carbol-fuchsin method for tubercle bacilli preparations and Pappenheim's pyronin-methyl green and Loeffler's methylene blue for the other preparations. With careful handling in the staining and washing, the celloidin membrane remains intact on the slide. Control preparations were made in connection with every chemical preparation.

For determining the action of ether, chloroform, and heat on bacteria it is obvious that the paraffin rings could not be used. In these experiments large quantities of bacteria were placed in vials and the ether and chloroform added. After four hours the ether and chloroform were permitted to evaporate considerably. The remains in the vials were then withdrawn with a pipette, placed on slides and permitted to evaporate to dryness. For the heat determinations the organisms were placed in vials with normal salt solution. The vials were then kept at a constant temperature in an incubator. After half an hour portions of the emulsion were withdrawn with a pipette and permitted to evaporate on slides.

The experiments recorded below were repeated a number of times. In some cases the results were not identical in one set of experiments. These differences in results were only slight and apparently of no particular consequence to the object of the experiments.

The main object in all of the experiments that follow was to determine the staining reaction of bacteria after treatment with chemicals and heat.

A. Action of alcohol on bacteria

Alcohol of various strengths was permitted to act on five different strains of bacteria for a period of five hours.

a. After 95 per cent alcohol. 1. Human tubercle bacilli. Stain the same as control, granules appear more distinct than in control.

2. Bovine tubercle bacilli. Stain the same as control, some crescent forms apparently not observed in control.

3. Bacillus megatherium (with spores). Bacilli stained fainter than controls, spores tinted.

4. Bacillus subtilis. Stained more intensely than control.

5. Unknown cocci and bacilli from a lymph-node culture, two strains of cocci, one intensely stained and the other very faintly in controls. The cocci appear to be destroyed. Two strains of bacilli (different in length) not observed in controls were intensely stained.

b. After 50 per cent alcohol. 1. Human tubercle bacilli. Some bacilli are very faintly stained, others appear to be slightly swollen.

2. Bovine tubercle bacilli. Some indication of disintegration, the bacilli intact were decidedly shrunken.

3. Bacillus megatherium. Bacilli could not be demonstrated by staining. The spores were decidedly swollen and in many parts of the field they were coalesced (partially dissolved).

4. Bacillus subtilis. Bacilli could not be demonstrated by staining. Field contained intensely stained granular debris.

5. Unknown cocci and bacilli from lymph-node culture. Field full of very minute well-stained cocci (granules?), also a few

large well stained cocci. Some of the larger cocci coalesced. Few exceedingly small well-stained bacilli. Large bacilli unstained.

c. After 25 per cent alcohol. 1. Human tubercle bacilli. Bacilli stain very faintly and appear shrunken. Granules in bacilli not visible.

2. Bovine tubercle bacilli. Great number of bacilli disintegrated.

3. Bacillus megatherium. Bacilli could not be demonstrated by staining. Some unstained bacillus-like forms partially coalesced. Spores coalesced, very few distinct in outline.

4. Bacillus subtilis. Granular debris, stained.

5. Unknown cocci and bacilli from lymph-node culture. Cocci could not be demonstrated by staining. Few small bacilli stained.

d. After 10 per cent alcohol. 1. Human tubercle bacilli. Bacilli appear more granular than control. Some disintegration.

2. Bovine tubercle bacilli. Most bacilli are granular, some crescent-shaped, some swollen, and some disintegrated. Some bacilli intact have a purple color.

3. Bacillus megatherium. Some unstained swollen bacilli present. Spores coalesced.

4. Bacillus subtilis. Field contains granular debris which has the appearance of minute cocci.

5. Unknown cocci and bacilli from lymph-node culture. Few intensely stained cocci, bacilli unstained.

e. After 5 per cent alcohol. 1. Human tubercle bacilli. Only a few bacilli intact and stained in thick part of smear, rest of field contains debris of disintegration.

2. Bovine tubercle bacilli. Some disintegration. Appear better preserved than after action of 10 per cent alcohol.

3. Bacillus megatherium. Bacilli could not be demonstrated by staining. Spores completely coalesced.

4. Bacillus subtilis. Only slight indication of a few unstained bacilli.

5. Unknown cocci and bacilli from lymph-node culture. Granular debris that appears like minute cocci. Few minute bacilli stained.

f. After 2 per cent alcohol. 1. Human tubercle bacilli. Almost completely disintegrated. Few swollen poorly stained bacilli present in field.

2. Bovine tubercle bacilli. Almost completely disintegrated. Few swollen poorly stained bacilli present in field.

3. Bacillus megatherium. Bacilli could not be demonstrated by staining. Spores coalesced.

4. Bacillus subtilis. Few intensely stained fragmented bacilli present in field.

5. Unknown cocci and bacilli from lymph-node culture. This preparation appears very much like the control. Bacilli apparently not stained.

B. Action of chloroform and ether on bacteria

a. After chloroform. 1. Human tubercle bacilli. Bacilli intact, but appear shrunken and more granular than control.

2. Bovine tubercle bacilli. Bacilli more faintly stained and appear more granular than controls.

3. Unknown bacilli, culture from intestinal contents of five-day-old kitten, two strains of bacilli, large and small. Large bacilli more granular than control, smaller forms clear and faintly stained.

4. Bacillus megatherium and spores. Few poorly stained and shrunken bacilli present. Spores not visible.

5. Staphylococcus albus. No normal cocci visible. Remains appear like exceedingly minute cocci.

b. After ether. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Remains, granular debris.

2. Bovine tubercle bacilli. Bacilli could not be demonstrated by staining. Remains, granular debris.

3. Unknown bacilli, culture from intestinal contents of five-day kitten. Bacilli could not be demonstrated by staining. Granular debris appears like minute cocci.

4. Bacillus megatherium and spores. Few disintegrated bacilli, remains mostly granular debris. Spores were not visible.

5. Staphylococcus albus. Remains, granular debris.

C. Action of acetic acid on bacteria

Acetic acid of various strengths was permitted to act on bacteria for a period of six hours. Glacial acetic acid was diluted with distilled water for the various dilutions.

a. After 0.5 per cent acetic acid. 1. Human tubercle bacilli. Bacilli disintegrated. Granular remains intensely stained (black).

2. Bovine tubercle bacilli. Many bacilli retain their form, others disintegrated. Intensely stained.

3. Unknown bacilli, culture from intestinal contents of kitten. Bacilli unstained, swollen, and coalesced.

4. Bacillus megatherium. Bacilli could not be demonstrated by staining. Spores swollen.

5. Staphylococcus albus. Swollen, unstained, and partially coalesced.

b. After 1 per cent acetic acid. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Remains granular, intensely stained.

2. Bovine tubercle bacilli. Bacilli could not be demonstrated by staining. Remains granular, faintly stained.

3. Unknown bacilli, culture from intestinal contents of kitten. Bacilli unstained, swollen, and distorted.

4. Bacillus megatherium. Bacilli unstained and distorted. Many forms contain 'bleb'-like swellings at end or center of bacillus, some contain two or three outpushings. Figure 7 is a free-hand drawing of a few of these bacilli.

5. Staphylococcus albus. Appear partially dissolved and coalesced. Unstained.

c. After 3 per cent acetic acid. 1. Human tubercle bacilli. Form of bacilli partially preserved. Faintly stained.

2. Bovine tubercle bacilli. Bacilli appear quite normal and well stained. Some appear to have vacuoles.

3. Unknown bacilli, culture from intestinal contents of kitten. Bacilli could not be demonstrated by staining. Remains, amorphous and faintly stained.

4. Bacillus megatherium. Few unstained bacilli that appeared partially dissolved. Spores coalesced.

5. Staphylococcus albus. Cocci unstained and coalesced.

d. After 5 per cent acetic acid. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Remains, minute, intensely stained granules.

2. Bovine tubercle bacilli. (Accidentally destroyed.)

3. Unknown bacilli, culture from intestinal contents of kitten. Bacilli could not be demonstrated by staining. Remains, amorphous and faintly stained.

4. Bacillus megatherium. Bacilli and spores unstained and coalesced.

5. Staphylococcus albus. Cocci unstained and coalesced.

D. Action of formaldehyde on bacteria

Formaldehyde of various strengths (diluted in distilled water) was permitted to act on bacteria for a period of six hours.

a. After 1 per cent formaldehyde. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Remains, intensely stained amorphous masses.

2. Bovine tubercle bacilli. Poorly stained bacilli that appear shrunken.

3. Unknown bacilli, culture from intestinal contents of kitten. Bacilli could not be demonstrated by staining.

4. Bacillus megatherium. Few unstained and swollen bacilli. Spores greatly swollen.

5. Staphylococcus albus. Cocci faintly stained and swollen.

b. After 3 per cent formaldehyde. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Remains intensely stained amorphous masses.

2. Bovine tubercle bacilli. Bacilli distorted in various ways: shrunken, crescent-shaped, and some with large intensely stained granules.

3. Unknown bacilli, culture from intestinal contents of kitten. No distinct bacilli stained. Unstained spore-like forms partially dissolved.

4. Bacillus megatherium. Bacilli could not be demonstrated by staining. Spores partially dissolved.

5. Staphylococcus albus. Cocci unstained and smaller than control.

c. After 5 per cent formaldehyde. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Remains, intensely stained, large granules.

2. Bovine tubercle bacilli. Bacilli could not be demonstrated by staining. Remains, granular.

3. Unknown bacilli, culture from intestinal contents of kitten. Some large and small bacilli present that stain more intensely than control, also some swollen and unstained forms.

4. Bacillus megatherium. Bacilli unstained and partially dissolved. Spores unstained and coalesced.

5. Staphylococcus albus. Cocci swollen, unstained, and coalesced.

E. Action of potassium bichromate on bacteria

Various strains of bacteria were subjected to the action of potassium bichromate in various concentrations for a period of four hours. The potassium bichromate was dissolved in distilled water.

a. After 0.5 per cent solution of potassium bichromate. 1. Human tubercle bacilli. Bacilli intact could not be demonstrated by staining. Remains, intensely stained granules.

2. Bovine tubercle bacilli. A few bacilli still retain form. Remainder of remains intensely stained granules.

3. Staphylococcus pyogenes aureus. Normal cocci could not be demonstrated by staining. Remains, intensely stained minute 'cocci.'

4. Bacillus megatherium and spores. Bacilli could not be demonstrated by staining. Spores swollen and stained.

5. Unknown cocci. Some cocci swollen and stained.

b. After 1 per cent potassium bichromate. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Granular remains intensely stained.

2. Bovine tubercle bacilli. Bacilli could not be demonstrated by staining. Granular remains intensely stained.

3. Staphylococcus pyogenes aureus. Cocci swollen and partially destroyed, also stained.

4. Bacillus megatherium and spores. Bacilli could not be demonstrated by staining. Spores unstained, some swollen.

4. Bacillus megatherium and spores. Bacilli and spores preserved, but unstained.

5. Unknown cocci. Few swollen and stained cocci. Clumps of stained granular debris.

c. After 2.5 per cent potassium bichromate. 1. Human tubercle bacilli. Bacilli intact could not be demonstrated by staining. Granular remains minute particles and intensely stained.

2. Bovine tubercle bacilli. Few faintly stained bacilli intact.

3. Staphylococcus pyogenes aureus. Cocci could not be demonstrated by staining. Granular remains.

4. Bacillus megatherium and spores. Few swollen and unstained bacilli. Outline of spores very faint.

5. Unknown cocci. Cocci very faintly stained and swollen.

F. Action of osmic acid on bacteria

Various strains of bacteria were subjected to the action of 1 per cent and 2 per cent osmic acid for a period of four hours.

a. After 1 per cent osmic acid. 1. Human tubercle bacilli. Bacilli well preserved, stain purple.

2. Bovine tubercle bacilli. Bacilli well preserved, stain deep red.

3. Staphylococcus pyogenes aureus. Cocci preserved, but unstained.

4. Bacillus megatherium and spores. Bacilli and spores preserved, but unstained.

5. Unknown cocci. Could not be seen on the slide.

b. After 2 per cent osmic acid. 1. Human tubercle bacilli. Poorer preservation than with 1 per cent osmic, faintly stained.

2. Bovine tubercle bacilli. Well preserved and intensely stained.

3. Staphylococcus pyogenes aureus. Cocci could not be seen on the slide.

4. Bacillus megatherium and spores. Bacilli and spores unstained, outlines difficult to see.

5. Unknown cocci. Preserved, but unstained.

G. Action of moist heat on bacteria

Various strains of bacteria were placed in vials containing physiological salt solution and kept in an oven at a constant temperature of 49°C.

a. After thirty minutes at 49°C. 1. Human tubercle bacilli. Bacilli could not be demonstrated by staining. Field apparently contained fat globules.

2. Bovine tubercle bacilli. Bacilli could not be demonstrated by staining. Field contained granular remains, stained amorphous masses and apparently fat globules.

3. Staphylococcus pyogenes aureus. Cocci could not be demonstrated by staining. Remains very minute granules.

4. Bacillus megatherium and spores. Bacilli could not be demonstrated by staining. Spores coalesced.

5. Unknown cocci. Cocci could not be demonstrated by staining. Remains, stained amorphous masses.

DISCUSSION

The results obtained from these experiments demonstrate that bacteria may lose their staining properties when subjected to the action of certain chemicals ordinarily used in microscopical technique. The degree to which the staining reactions were affected varied with the different chemicals and also with the strain of bacteria.

In many cases the bacteria retained their form, but were unstained, and in other experiments the bacteria were fragmented. In the cases where the organisms could not be seen they apparently had been dissolved or fragmented. In the majority of experiments where the remains on the slide were granular and fragmented these remains were stained. The possibility suggests itself that mitochondria may behave in the same way and that some of the irregularly shaped mitochondria sometimes observed may be the fragments resulting from chemical action.

The visibility of unstained bacteria varies with the difference in refraction of the bacteria and the surrounding medium. In those cases where the bacteria could not be seen the granular remains indicated the destruction of the organism. Unstained bacteria lodged in the cytoplasm of tissue cells cannot be distinguished easily and in some cases they are not visible. It is generally supposed that mitochondria are dissolved by the action of certain chemicals. It is possible that in many cases where they cannot be demonstrated by staining their form has been retained, but unstained and consequently not readily observed.

Bacteria apparently respond to heat in the same way that mitochondria do. The end-product from the action of heat was not the same for all the strains of bacteria that were used for this experiment. In some cases the remains were granular, in others they were amorphous. The amorphous material apparently represented the residuum of a solution after evaporation.

Cowdry ('18, p. 68) has noted the presence in some secreting cells of mitochondria with 'bleb-like' swellings and in egg cells of 'dumb-bell-shaped' mitochondria. The action of 1 per cent acetic acid on Bacillus megatherium is significant in this connection. The imitation of such 'bleb-like' and 'dumb-bell-shaped' mitochondria by bacteria as the result of chemical action suggests the possibility that mitochondria of these types may be due to the action of the chemicals used in fixation.

CONCLUSIONS

The results obtained in subjecting bacteria to mitochondrial staining methods and to the chemicals that have been utilized to determine the chemical nature of mitochondria appear to demonstrate that these methods are not specific for mitochondria, but have a similar reaction on bacteria. *To the degree that these staining methods and chemical reactions are not specific, bacteria and mitochondria have a similar chemical constitution.*

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PLATE 1

EXPLANATION OF FIGURES

All the figures, with the exception of figure 7, were made with the aid of the camera lucida. They were all drawn to the same scale. The lenses used were: 2-mm aproch. oil-immer. obj., comp. ocular no. 8.

1 Bacillus subtilis from an old culture one hour after the application of janus green.

2 Bacillus subtilis, *a*) 5½ hours after application of janus green; *b*) 20 hours after application of janus green.

3 Bacillus subtilis from a 48-hour culture 24 hours after application of janus green.

4 Bacillus megatherium from an old culture 5½ hours after application of janus green.

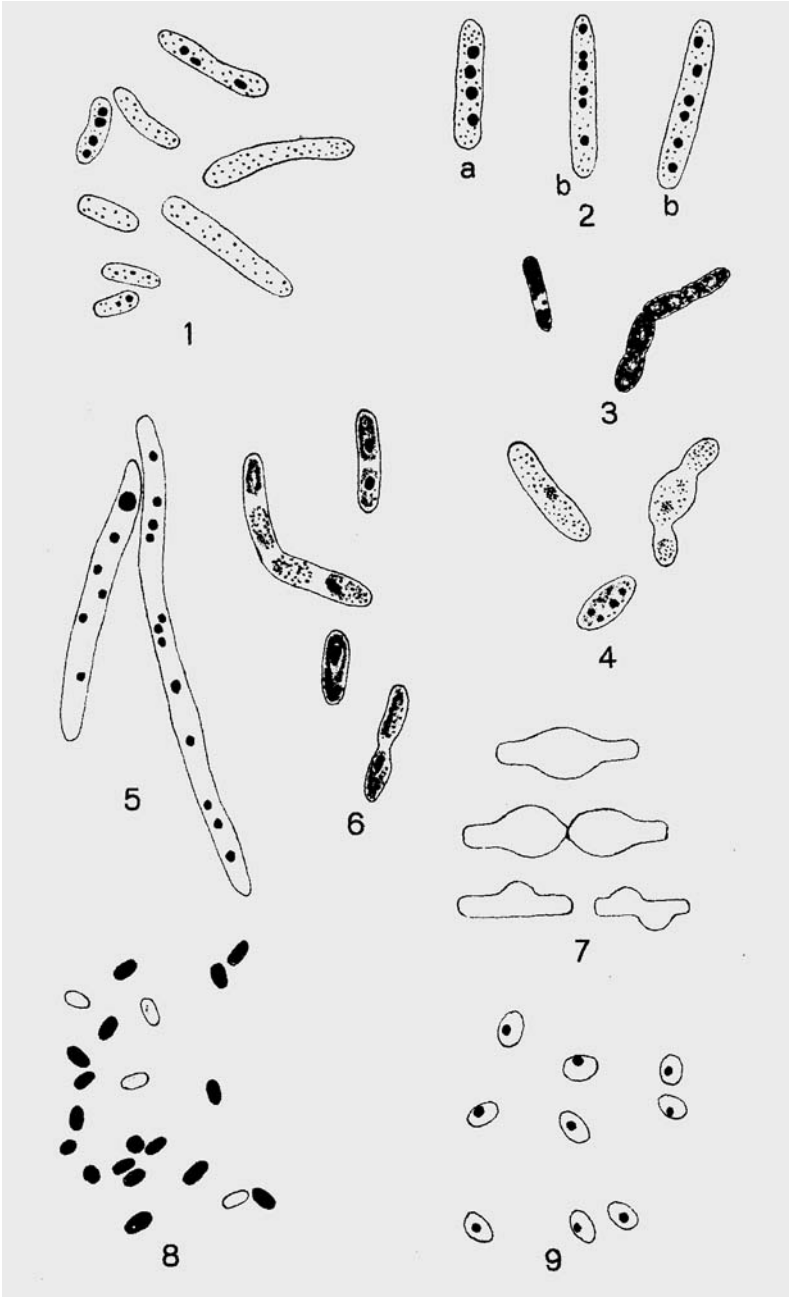
5 Bacillus megatherium from a 20-hour culture 15 minutes after application of janus green.

6 Bacillus megatherium from an old culture ½ hour after application of janus green.

7 Free-hand drawing of bacillus megatherium after action of 1 per cent acetic acid for a period of six hours.

8 Bacillus coli 15 minutes after application of janus green.

9 Bacillus coli (same specimen as in fig. 8) 5½ hours after application of janus green.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ADDENDUM

After this paper was submitted for publication, my attention was called to a work by Portier ('18) entitled, "Les Symbiotes," and to criticisms of Portier's book by Regaud ('19) and Guilliermond ('19).

Unfortunately, I have not been able to secure a copy of Portier's book in time to review it in this article. However, I have perused Regaud's and Guilliermond's criticisms. From these criticisms it is apparent that Portier in 1918 stated a theory regarding mitochondria that coincides with a conception of these bodies that has been growing in my own mind. I was not ready to state this hypothesis until I had collected more evidence in its support. A brief consideration of Regaud's and Guilliermond's criticisms is pertinent at this time.

Obviously, the details of Portier's evidence cannot be considered in this discussion. Regaud quotes the following resumé from Portier's "Les Symbiotes": "Chaque cellule vivante renferme dans son protoplasme des formations que les histologists désignent sous le nom de mitochondries. Ces organites ne seraient pour mio autre chose que des bactéries symbiotiques, ce que je nomme des symbiots. . . . . La bactérie symbiotique vient du milieu extérieur: elle peut, dans certains cas, y retourner et vivre d'une vie indépendante. Les bactéries seraient donc les seuls êtres simples; tous les autres seraient doubles."

Regaud indicates various characteristics of mitochondria that are supposedly not shared by bacteria. He mentions the inconstancy of form of mitochondria, their behavior towards acids and metallic salts, their albumin-lipoid constitution, their fragility, the impossibility of mechanical extraction of mitochondria from the living cell, and the synthetic properties of mitochondria. He also indicates the following characteristics of bacteria that presumably, are not shared by mitochondria: bacteria are definite organisms having a stable form (difficult to change in shape); bacterial life is generally resistant to chemical and physical agents; bacteria are easily extracted mechanically from living cells without alteration of the form of the bacteria; the form and structure of bacteria and even their staining qualities are indifferent to fixation.

I shall discuss briefly each of these characters that Regaud considers distinctive.

1. Inconstancy of form of mitochondria. This cannot seriously be considered a characteristic of mitochondria. It is a well established fact in bacteriological technique that certain bacteria assume different forms in different media (Jordan, '20, p. 67).

2. The behavior of mitochondria towards acids and metallic salts. In the second section of this paper I have given sufficient evidence that

some bacteria behave like mitochondria to acetic acid and other chemicals. The behavior of bacteria after fixation with a fixative containing potassium bichromate is definitely indicated by their staining reaction and is not unlike mitochondria.

3. The albumin-lipoid constitution of mitochondria. The chemical nature of mitochondria is unknown. I have shown in the second section of this paper that the chemical reactions used in attempting to determine the chemical nature of mitochondria have a similar effect on some bacteria.

4. The fragility of mitochondria. Mitochondria vary in fragility. This, I believe, has been assumed by various investigators. In a paper in preparation I will definitely demonstrate this difference. This character of mitochondria, per se, is in favor of a bacterial nature of mitochondria. Biological data furnish many examples of plants and animals that have become 'fragile' as a result of well-developed symbiosis and parasitism. The tapeworm is an example of a comparatively fragile organism. Its relationship to host is not as intimate as an intracellular symbiotic bacterium would be to its host. Surrounded by a living cytoplasmic environment, it is to be expected that a well-established symbiotic organism would lose many properties that the genetic type possessed. Further, one would expect the symbiotic form to acquire new properties.

5. The impossibility of mechanical extraction of mitochondria from the living cell. This apparent difficulty is undoubtedly dependent upon the fragility of mitochondria and consequently is irrelevant to the real problem.

6. The synthetic nature of mitochondria. Regaud argues that mitochondria are unlike bacteria in that they exhibit synthetic properties in the cytoplasm. He quotes himself and other investigators to support the contention that mitochondria produce secretion granules, pigment, etc. It appears to me that Regaud's argument is at least, equally convincing evidence that mitochondria are organisms.

7. Bacteria are definite organisms having a stable form. Bacteriological evidence does not support this contention. "The tubercle bacillus, for example, under ordinary conditions, is a typical rod, but sometimes produces branching filaments, and has been placed by some writers with the *trichomycetes*" (Jordan, '20, p. 64).

8. Bacterial life is generally resistant to chemical and physical agents. The chemical and heat experiments recorded in the second section of this paper refute this statement. Bacteria are not only affected by these agents, but in some cases are extremely sensitive to them.

9. Bacteria are easily extracted mechanically from living cells without alteration of the form of the bacterium. This argument has no bearing on the problem for, a priori, it must be admitted that a bacterium that develops an intracellular symbiotic existence would acquire fragility.

10. The form and structure of bacteria and even their staining qualities are indifferent to fixation. This is not in agreement with the results recorded in the second section of this paper. It was found that not only the form was altered by the fixation, but the staining qualities were also distinctly altered. In the paper in preparation I shall give further evidence regarding this point.

Regaud states that absolute differences between bacteria and mitochondria are incontestable. Further, he demands that if Portier will not admit that there are differences, he will have to demonstrate that these two structures (mitochondria and bacteria) can change from one to the other. I cannot find in Regaud's criticism the 'absolute differences' which he claims are incontestable. Regarding the demand for a demonstration of reversibility of mitochondria and bacteria as a proof that mitochondria are organized entities, it seems to me that one has just as much ground to demand that it be demonstrated that a tapeworm can revert to a free-living organism to establish its individuality.

Portier ('19) answers the arguments advanced by Regaud. Not having a full knowledge of Portier's data, I shall not attempt to consider Portier's rebuttal. However, one argument advanced by Portier in explaining the source of his 'symbiotes' (mitochondria) invites a critical consideration. Portier found bacteria in the intestine of the rabbit and apparently found similar bacteria in the cytoplasm of the intestinal epithelial cells. From this observation Portier concludes that the source of 'symbiotes' is from the intestinal contents. Regaud, justly, refuses to accept this interpretation of the phenomenon. I have observed the same phenomenon in the intestinal contents and the intestinal epithelial cells in a one-day-old kitten after mitochondrial fixation and staining. The fact that mitochondria may be demonstrated in the cells of embryos before the intestine is formed excludes the possibility of such an origin. The question of the origin of mitochondria is a major problem that may well rest until the nature of mitochondria has been established.

Guilliermond ('19) discusses three sections of Portier's book. I shall briefly discuss his criticisms in the order given.

1. Analogous forms. Guilliermond admits the analogy of form and further admits that mitochondria exhibit the property of division. He calls attention to the fact that the slightest upset in osmotic equilibrium suffices to change the character of mitochondria. In hypotonic media mitochondria immediately swell and transform into large vesicles. Of most important value as evidence, he argues that mitochondria have very little resistance to alcohol, chloroform, and acetic acid, and that it has been shown that a temperature of 40°C.[1] is sufficient to destroy the mitochondria in a few moments. He also says: "Up to the present time bacteria are not known that exhibit such fragility."

[1] In a correction (Compt. rend. des Soc. Biol., T. 82, p. 396), Guilliermond changes the temperature at which mitochondria disappear to 47°C.

This criticism deals with the fragility of mitochondria. I have answered this criticism above. However, I again insist that even if such a difference were a reality, it has no bearing on the problem. It must be admitted, on the basis of known biological behavior, that fragility is an accompaniment to well-established symbiosis and parasitism.

2. Bacteria are stained like mitochondria by Regaud's method. Portier's 'symbiotes' resist alcohol and acetic acid, are stained easily without change after fixation. Guilliermond states that this fact is an excellent means of differentiating between mitochondria and 'symbiotes.' Guilliermond admits that some mitochondria are more resistant than others to acetic acid and alcohol, but maintains that the more resistant forms are no longer mitochondria, but plastids differentiated from mitochondria.

In the first section of this paper I have shown that bacteria are stained like mitochondria by a number of mitochondrial methods, including the vital janus green method. From Guilliermond's criticism it would appear that Portier's 'symbiotes' are not mitochondria, but some other organism. In my work on staining of bacteria with mitochondrial methods I have found no fundamental difference in the staining reactions of mitochondria and bacteria. Guilliermond's statement that the more resistant mitochondria are no longer mitochondria needs elucidating evidence. If mitochondria metamorphose into organs that exhibit synthetic properties, then they assume properties that are characteristic of organisms. Numerous investigators have observed that mitochondria differ in their power of resistance to acetic acid and alcohol. I have shown in the second section of this paper that bacteria also differ in their reactions to these chemicals.

3. Mitochondria may be cultivated in certain cases. Guilliermond does not accept Portier's statement that he has grown mitochondria. He concludes with the statement: "We cannot conceive that anyone can culture such fragile elements."

I am not in a position to intelligently consider this latter criticism at this time. On the basis of evidence that I shall submit in a paper in preparation, I feel confident that mitochondria may be transferred intact to culture media. While it must be admitted that a demonstration of mitochondria growing as independent organisms in a culture medium would be absolute proof of their organized or bacterial nature, *the lack of such a demonstration is not proof that they are cytoplasmic organs and not organisms.*

The writer will discuss Regaud's and Guilliermond's criticisms at more length in a paper in preparation. From the preceding discussion, it is apparent that the fundamental aspects of the problem are not clearly defined. This confusion is apparently due to the vagueness of mitochondrial and bacterial definitions. Mitochondrial literature has not supplied a satisfactory definition of mitochondria. Jordan's "General Bacteriology" does not contain a definition of bacteria.

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