BACTERICIDAL ACTION OF OLEIC ACID FOR TUBERCLE BACILLI[1]

II. Morphological Response

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In the previous report (Minami, 1957), the bactericidal action of oleic acid on tubercle bacilli was studied, and it was concluded that some particular portion of the cell's surface structure was concerned with this action. In the present paper, the electron microscopic studies were carried out to look for a morphological base for the bactericidal action of oleic and other homologous fatty acids on tubercle bacilli.

MATERIALS AND METHODS

Mycobacterium avium (TAKEO strain) was used throughout the present studies. The 3-day cultures of this strain grown in "tween"-containing liquid synthetic medium (Minami, 1957) were collected by means of centrifugation, washed with distilled water two or three times, and then treated with sodium oleate, linoleate, or stearate at various concentrations in phosphate buffer (pH 6.8, M/40) for 1 or 20 hr at 37 C. The treated cells were collected, washed three times, and finally suspended in distilled water at a suitable concentration. The washed cell suspension was then dropped on the copper mesh, which was covered with collodion membrane before use. The bacilli were dried at room temperature and chromium shadow-cast was undertaken. The electron micrographs were taken by using the HU-6 type electron microscope (magnetic type, 50 kv), Hitachi Co., in the Central Laboratory, Fukushima Medical College, at a primary magnification of 3,500 ×.

RESULTS

Effects of contact with sodium oleate at various concentrations for 1 hr. The washed cells of tubercle bacilli were treated with 10^{-2}, 10^{-3} and 10^{-4} M oleate, respectively, for 1 hr at 37 C and prepared for electron microscope observations. In general, the morphological alteration due to oleate was accelerated by increasing its concentration.

When the bacilli were treated with 10^{-4} M oleate, few morphological changes were found in their electron micrographs (figure 1, figure 8), although in some cells the small electron transparent areas (ETA) were occasionally found just inside the cell wall (figure 7).

The ETA were also formed in almost all the cells treated with 10^{-3} M oleate, at which concentration the ETA increased considerably in size and number, and it was further observed that the ETA tended to be located at a regular interval of the cell bodies (figures 4–6). It is noteworthy that these ETA were already formed before the shadow-cast was undertaken since the shadow was cast over all the parts of the ETA (figures 4–6). The shadow line proves that ETA were thinner than the other cell portions (figure 6). In some cells, the picture was characterized by an indistinct and lytic cell surface (figure 5).

When treated with 10^{-2} M oleate, a large portion of the cell population had a tendency to lyse, and many small granules were found to adhere around the periphery of these cells (figure 2). The ETA were formed in the remaining cells (figure 3). Of course, the nontreated cells had no ETA and no changes were found in their structures (figure 1).

Effect of contact with oleate for 20 hr. The cells were treated with 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} M oleate for 20 hr and their electron micrographs are summarized in figures 9–11. The morphologically discernible action of oleate was generally accelerated more markedly than in the cases of only 1 hr of contact.

In the cells treated with 10^{-5} M oleate, it was difficult to find any ETA developed, but one or two small ETA were occasionally found in a few cells. By treating with 10^{-4} M, ETA were more frequently formed, although most of the cell population did not have any ETA (figure 11). In the case of 10^{-3} M oleate, the cytoplasmic

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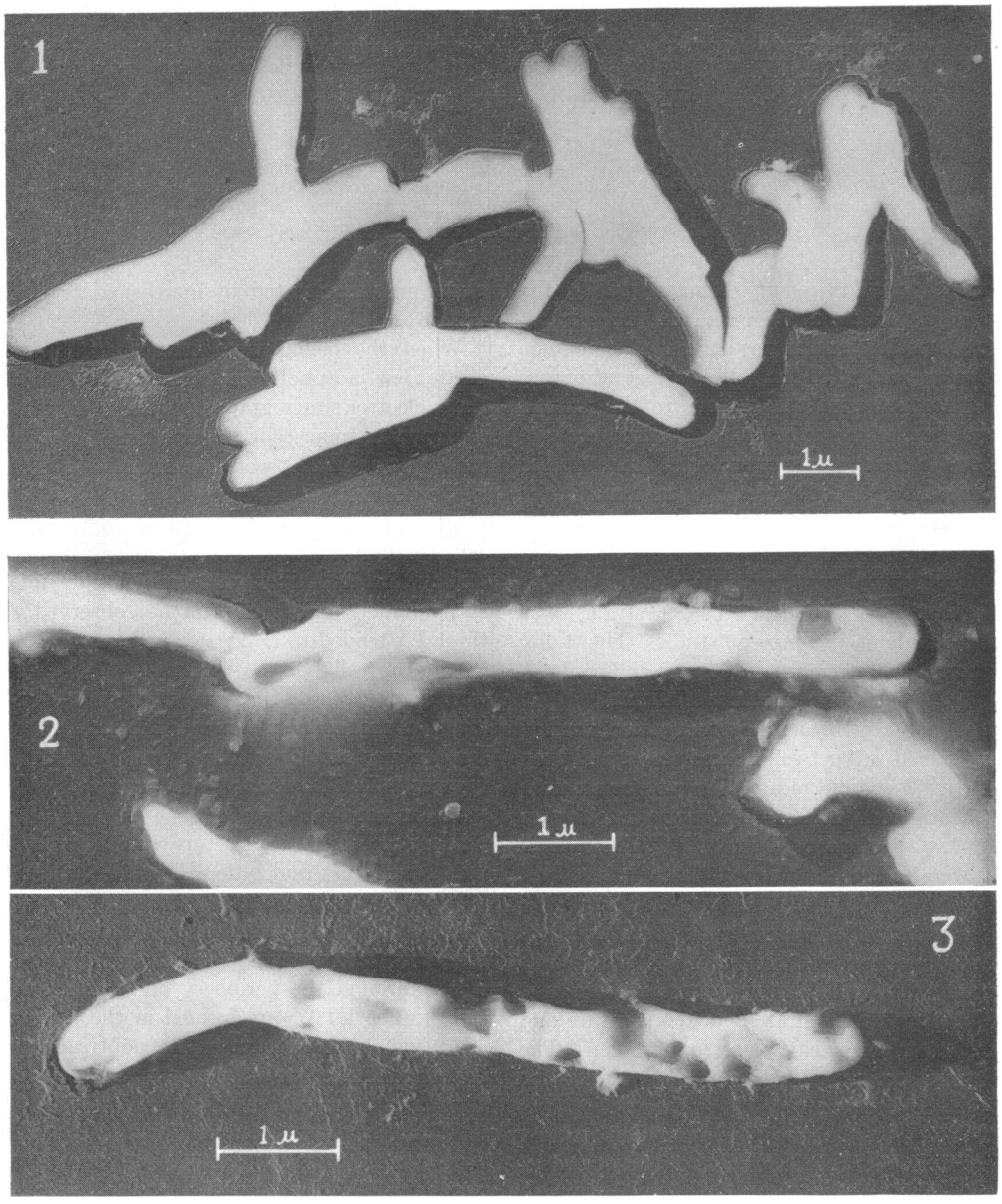
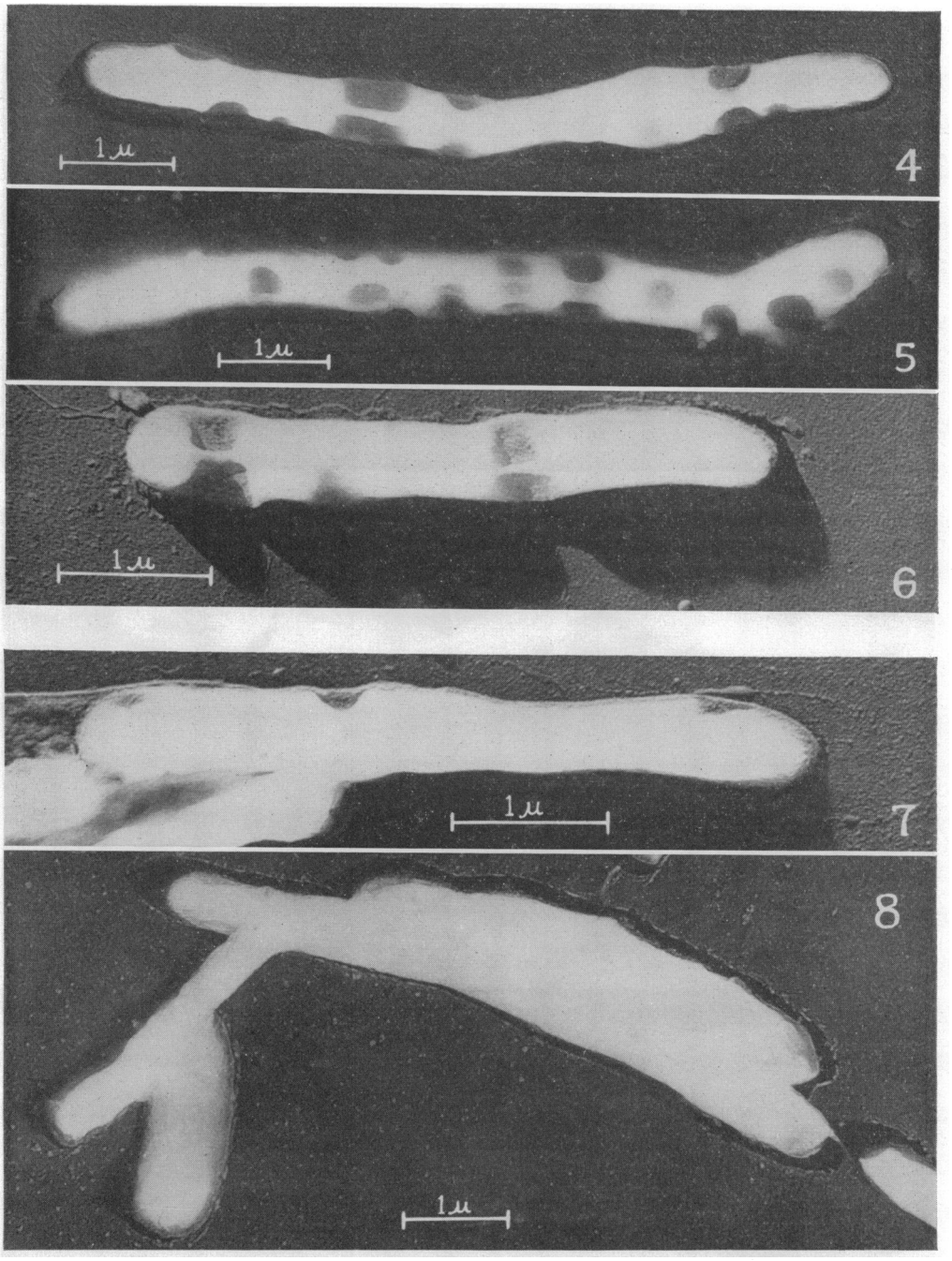


Figure 1. Electron micrograph of *Mycobacterium avium* (TAKEO strain), without any treatment, shadow-cast with chromium.

Figures 2-3. Mycobacterium avium treated with 10^{-2} M oleate for 1 hr.

materials were cut off deeply by ETA, and it was difficult to find any intact cells (figure 10). After treating with 10^{-2} M oleate for 20 hr, the majority of the cells lysed, and intact cells were difficult to observe (figure 9).

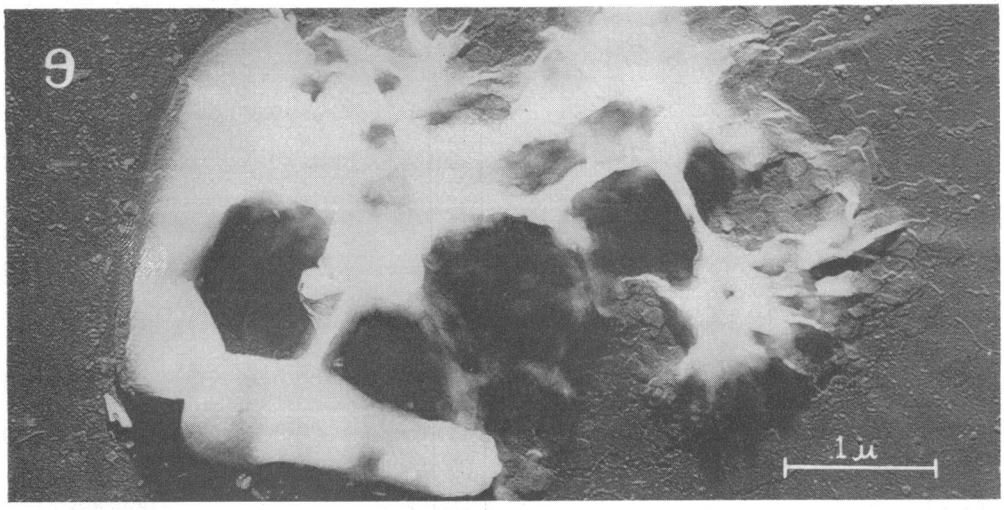
Effect of linoleate. By treating with 10^{-3} M linoleate for 1 hr, ETA were formed at nearly the same position in the cell and also with nearly the same frequencies as in the case of oleate, but they seemed to show somewhat different appearances from that of oleate. After treatment for 20 hr almost all the cells were ruptured (figure 12) and ETA formation was conspicuous in a few remaining cells (figure 13). The position of ETA

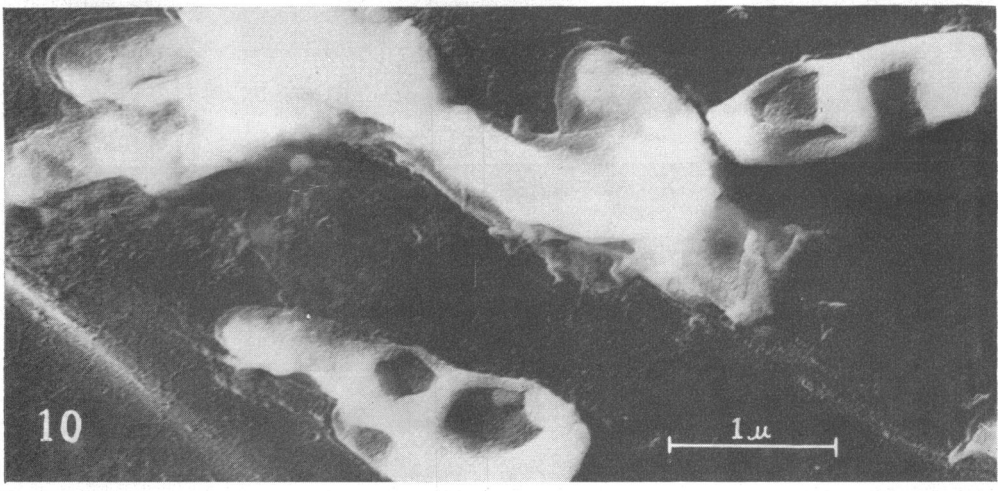


Figures 4–6. Mycobacterium avium treated with 10^{-3} M oleate for 1 hr.
Figures 7–8. Mycobacterium avium treated with 10^{-4} M oleate for 1 hr.

would be the same as that of oleate, but their shape was markedly different from that of the cells treated with oleate.

Effect of sodium stearate. The bacilli treated with 10^{-3} M of sodium stearate for 1 hr had no ETA, while after 20 hr of contact most of the cytoplasmic materials disappeared from the cells and ghostlike cells increased in number. As shown





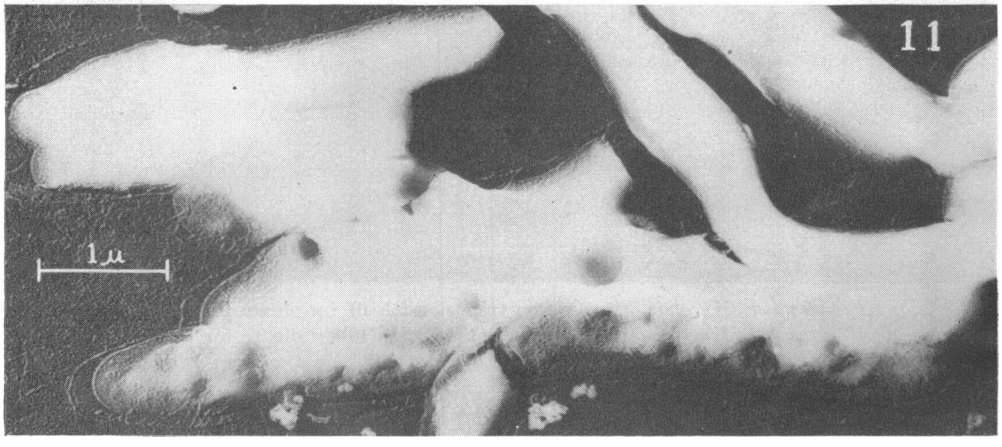
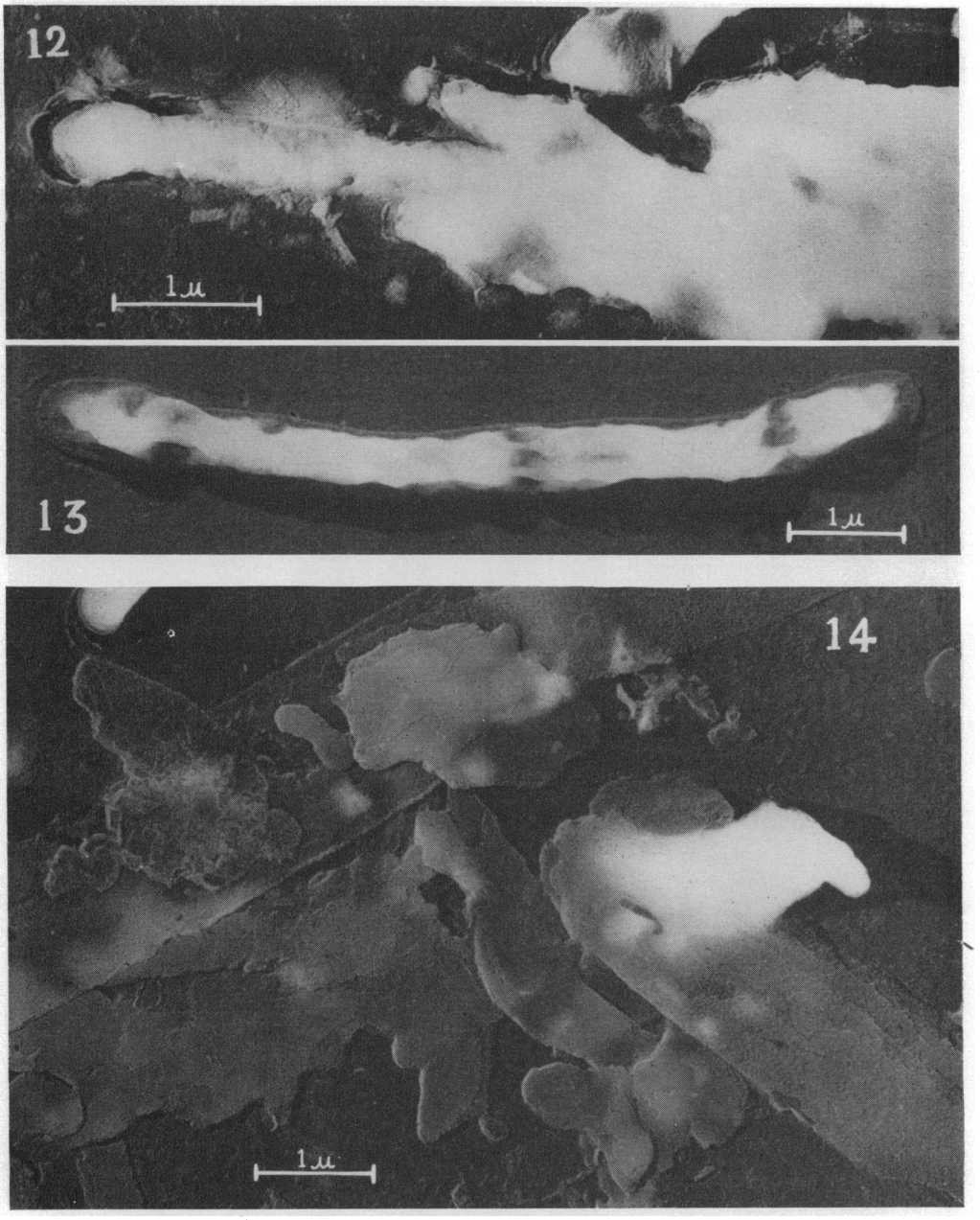


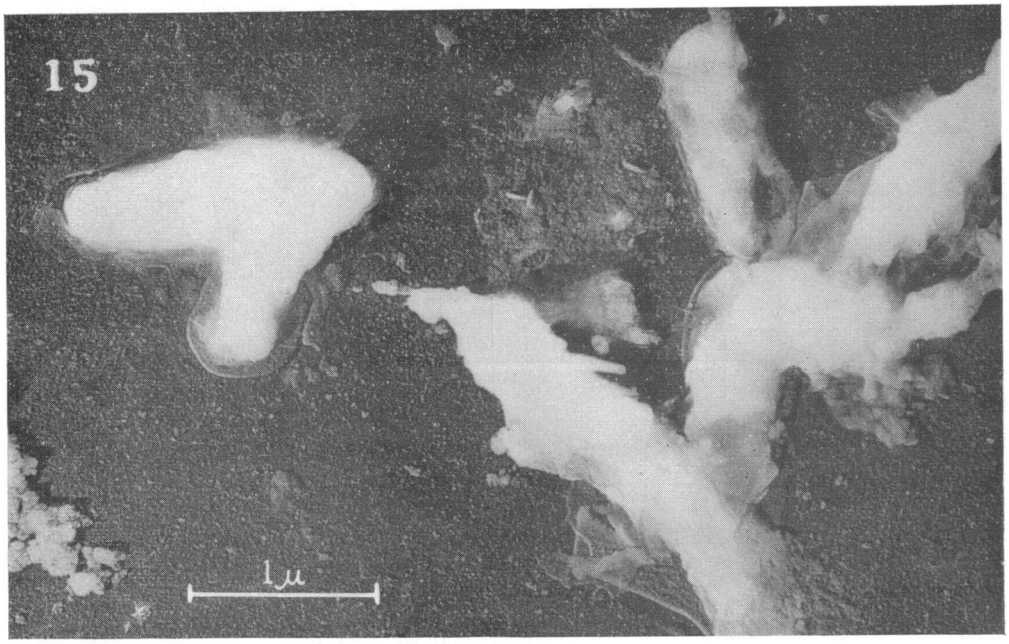
Figure 9. Mycobacterium avium treated with 10^{-2} M oleate for 20 hr.
Figure 10. Mycobacterium avium treated with 10^{-3} M oleate for 20 hr.
Figure 11. Mycobacterium avium treated with 10^{-4} M oleate for 20 hr.



Figures 12–13. Mycobacterium avium treated with 10^{-3} M linoleate for 20 hr.
Figure 14. Mycobacterium avium treated with 10^{-3} M stearate for 20 hr.

in figure 14, it seems that these transparent nonstructural bacilli consist only of cell membrane without their cytoplasmic materials.

Effect of organic solvents. After treating with acetone, ether or ethanol for 1 hr at 37 C, ETA were not formed, although the cell structure was markedly destroyed and shrunken. Figure 15 shows the electron micrograph of the cells treated with ether. On the other hand, ETA was formed by treatment with petroleum ether for 20 hr (figure 16) although noticeable changes were not observed at contact for 1 hr.



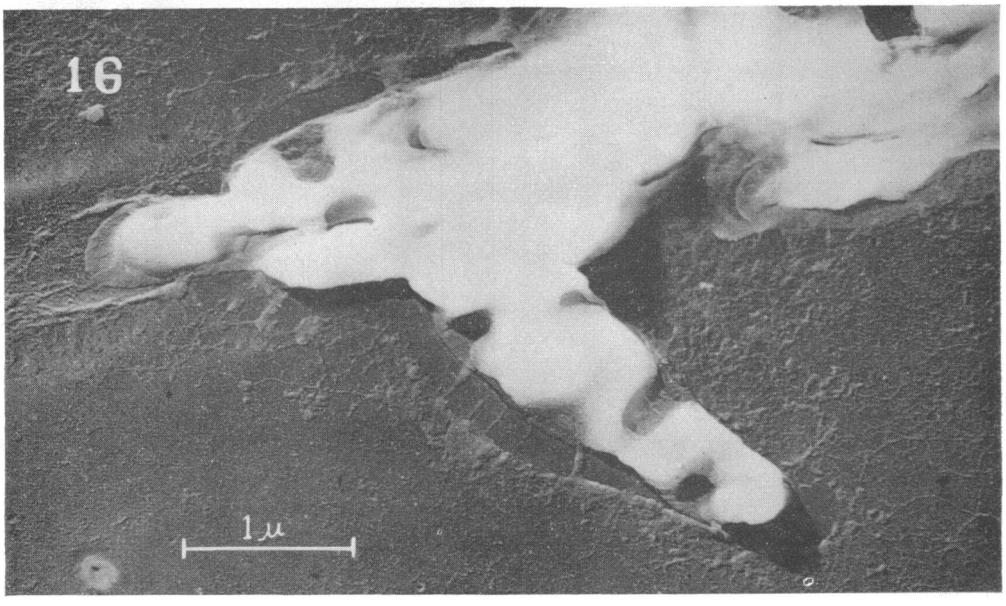


Figure 15. Mycobacterium avium treated with ether for 1 hr.
Figure 16. Mycobacterium avium treated with petroleum ether for 20 hr.

DISCUSSION

It is concluded from the present electron microscopic studies that electron transparent areas (ETA) are characteristically formed just inside the cell wall of the tubercle bacilli which are treated with oleate or linoleate.

The tubercle bacilli, including the avian type, usually have electron transparent vacuoles as a normal structure of the cells (Knaysi *et al.*, 1950), and recently Brieger *et al.* (1954) demonstrated that the vacuoles develop in the cells at a definite stage of their life cycle. The ETA which are

found in the present studies are different from such vacuoles, since (1) they are the result of treatment with oleate or linoleate, (2) they are independent of the life cycle of the bacilli,[2] and (3) they differ in form and position. On the other hand, it is reported by many investigators (Lembke and Ruska, 1940; Wessel, 1942; Knaysi *et al.*, 1950; Mudd *et al.*, 1951; Takeya *et al.*, 1954; Shinohara, 1954) that the cells of tubercle bacilli are vacuolized in the electron microscope by intense electron bombardment for short periods. The ETA, however, are essentially different from the vacuoles caused by the electron beam. The shadow-cast proves decisively that ETA are formed before the cast is made.

It is claimed that the bactericidal activity and the inhibition of oxygen uptake of the human type of tubercle bacilli are increased by increasing the number of the unsaturated bonds of long-chain higher fatty acids, such as stearate, oleate linoleate, and linolenate (Boissevain, 1926; Bergström *et al.*, 1946). In the present observations, the same tendency is confirmed, *i.e.*, the ETA are formed by treating with oleate and linoleate, but not with stearate. Stearate, however, is very interesting in another sense, as the cell structures are mostly disrupted and only ghostlike cells, or cell membranes, remain in fields of specimens treated with 10^{-3} M for 20 hr. To determine whether the ETA are formed by the solvent action of the fatty acids, the bacilli treated with ethanol, ether, or acetone were examined in the electron microscope, but ETA did not develop in these treated cells. Shinohara (1956, personal communication) suggests that similar ETA are observed in the bovine type of tubercle bacilli treated with petroleum ether. Cells treated for 1 hr with petroleum ether showed no morphological changes, but the ETA-like pictures were taken in the specimens treated for 20 hr. It would be difficult to decide whether such a lipoid-extracting action of petroleum ether is the main cause of the ETA formation by oleate and linoleate. Therefore, at the present stage of the studies, the mechanisms by which these unsaturated fatty acids are concerned with the bactericidal action and the ETA formation of the tubercle bacilli are quite unknown.

[2] The ETA or ETA-like structures were not observed in the cells cultivated for 3, 6, or 12 hr or for 1, 2, 3, 4, or 7 days, if they were not treated with oleate or linoleate.

The lytic action of oleic acid to tubercle bacilli has been described by McJunkin (1923) and Boissevain (1926). In the present studies, it is shown that the bacilli lyse after being treated with a higher concentration of oleate (10^{-2} M) and in some cases many small granules are found to adhere to the periphery of the lysed bacilli. These granules seem to be some normal components of the cytoplasmic material of the bacilli.

In the previous report (Minami, 1957), it is concluded that the bactericidal action of oleic acid against tubercle bacilli is concerned with their specific surface structure. The bacilli are killed by far lower concentrations of oleate (10^{-5} M or less). The present electron microscopic studies could not prove any significant morphological changes of the bacilli at this concentration of oleate. The metamorphical tendencies caused by higher concentrations, however, are clearly characterized by the specific ETA formation at 10^{-3} M and lytic action at 10^{-2} M. Thus, it seems that the morphological basis of bactericidal action of oleic acid would be the tendency to form ETA, although any visible ETA are not found at its critical concentration for bactericidal action. Such ultramicro structural changes would not be expected to be visible by the present techniques in which the *whole cells* of the bacilli are used by themselves for preparing the specimens.

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SUMMARY

To seek the morphological basis of bactericidal action of oleate against tubercle bacilli (avian type), electron microscopic studies were carried out. The particular electron transparent areas (ETA) were found to develop in the cells treated with higher concentrations of oleate and linoleate (10^{-3} M), although no significant morphological changes were found at lower concentrations (10^{-5} M), at which the cells were killed. The nature of ETA and their significance for the bactericidal action of oleate were discussed.

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