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Effect of Oleic Acid on Growth and Cell Structure of Mycobacteria

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ABSTRACT

SCHAEFER, WERNER B. (National Jewish Hospital, Denver, Colo.), AND C. WILLARD LEWIS, JR. Effect of oleic acid on growth and cell structure of mycobacteria. J. Bacteriol. **90**:1438–1447. 1965.—The growth-stimulatory effect of fatty acids on *Mycobacterium kansasii* and other mycobacterial species is associated with transient changes in the morphology of the bacteria. One change observed is the appearance of intracellular vacuoles separated by chromatinic crossbars. Evidence is presented that these changes are due to the rapid metabolic uptake and the accumulation of lipid in the form of globules. This process provides the cells with an internal reservoir of a preferred carbon and energy source. In certain mycobacterial species, including *M. kansasii*, the morphological changes are elicited by free and by esterified fatty acid; in others, only by free fatty acid. The latter strains apparently lack the enzyme to split the fatty acid ester.

The stimulatory effect of long-chain fatty acids on the growth of *Mycobacterium tuberculosis* and other mycobacteria is well known (Dubos and Davis, 1946; Dubos, 1947; Schaefer, 1952; Schaefer and Mandler, 1958; Hedgecock, 1962), but very little is known about its physiological basis. The possibility of gaining some insight into the mode of action of fatty acids on mycobacteria appeared when it was observed that *M. kansasii* (Subcommittee on Mycobacteria, 1962), *M. balnei* (marinum) (Linell and Norden, 1954), and some skotochromogenic mycobacteria not only showed enhanced growth on fatty acid-containing media but also striking changes in their cellular morphology during the early growth phase. Alternating stained and unstained segments were observed to appear, giving the cells a ladderlike, cross-barred aspect (Schaefer and Mandler, 1958).

This paper describes these changes more fully and attempts to elucidate their connection with the mode of action of fatty acids on the growth of mycobacteria.

MATERIAL AND METHODS

The strain of *M. kansasii* used was isolated from the sputum and the resected lung tissue of a patient with pulmonary disease resembling tuberculosis. It was cultivated in liquid medium having an ammonium salt or sodium glutamate as the nitrogen source, and glucose or glycerol as the carbon source (Schaefer, Marshak, and Burkhart, 1949). Bovine albumin fraction V was added as a detoxifying agent. Tween 80 (polyoxyethylene, sorbitan monoleate; Atlas Powder Co., Wilmington, Del.), or oleic acid-albumin complex (Dubos and Middlebrook, 1947) was used as the source of fatty acid. Cultures were incubated at 37 C in screw-capped tubes (20 by 125 mm) on a roller tube apparatus revolving at 20 rev/min. Growth was measured twice daily at a wavelength of 650 mμ in a Coleman Junior spectrophotometer.

For study of the bacteria on solid media, petri dishes with oleic acid-albumin-agar were used (Dubos and Middlebrook, 1947). The media were inoculated by means of a spreader using a turntable, and the morphology of the bacteria at various times of growth was studied by impression smears obtained by cutting small agar blocks with a dissecting knife, impressing the inoculated side on a glass slide, and removing the agar. The air-dried smears were fixed by formalin vapors in a Coplin jar for 10 min. The slides were stained without heating by a modified Ziehl-Neelsen stain containing Tween 80 (Aubert, 1950).

For the differentiation of the cell structures, the following selective staining methods were used. Cell walls and nuclear chromatin were examined after treatment of the preparations according to the methods of Robinow (1949). Lipids were stained with a 0.02% solution of Sudan black B in 70% ethyl alcohol (Burdon, 1946). For the identification of carbohydrates, the periodic acid-Schiff stain was used.

The living and unstained bacteria were exam-

ined in a phase microscope. Photomicrographs were taken with an AO research microscope equipped with 90× oil-immersion apochromatic objective, 10× compensating eye piece, and AO-ortho-illuminator. A green filter and Kodak Panatomic-X film were used. The magnification on the prints was 3,000-fold.

For electron microscopy, the bacteria were introduced into liquid medium and distributed into screw-capped tubes in 7-ml portions. The tubes were incubated on the roller tube apparatus at 37 C. At chosen times, the bacteria were collected by centrifugation, and the sediments were combined and suspended in 3 ml of a 1% solution of osmium tetroxide in acetate-Veronal buffer of *p*H 6.1 containing 0.1 M $CaCl_2$, and 0.1 ml of 10% tryptone (Ryter et al., 1958). After fixation for 2 hr at 2 C, the suspended bacteria were dehydrated rapidly, by several changes in acetone of increasing concentrations, and were then embedded in Araldite-Epon in the tips of no. 5 gelatine capsules. Ultrathin sections were made, by use of a diamond knife and a Porter Blum MT-1 ultramicrotome. They were poststained with a saturated solution of uranyl acetate in 50% ethyl alcohol, washed in distilled water, and examined in a Phillips EM-200 electron microscope.

RESULTS

Effect of fatty acid on growth. The growth of *M. kansasii* in a medium with Tween 80 as the source of fatty acid and glycerol as the second carbon source and in media with Tween or glycerol as the sole carbon sources is shown in Fig. 1. The inoculum was a 100-fold dilution of a fully grown culture in Tween-albumin medium. In the presence of Tween growth started rapidly, whereas in the absence of Tween and with glycerol as the sole carbon source growth became rapid only after a period of 5 days. In the media with Tween as the sole carbon source, the initially rapid growth soon became arrested at a level roughly proportional to the amount of Tween initially added. In the presence of glycerol as a second carbon source, growth continued unchecked. Although not shown in the figure, the stimulatory effect of fatty acid was also observed if it was supplied as an oleic acid-bovine albumin complex. Surface growth also was more rapid on oleic acid-albumin agar medium than on the same medium without oleic acid.

Effect of fatty acid on cell structure. The morphological changes of the bacteria during growth on oleic acid-albumin-agar medium are illustrated by a series of impression smears made at various times of incubation and stained by the acid-fast stain (Fig. 2). At the time of inoculation, the bacteria which came from a 7-day-old culture in Tween-albumin medium were uniformly stained or had one or two deeply stained

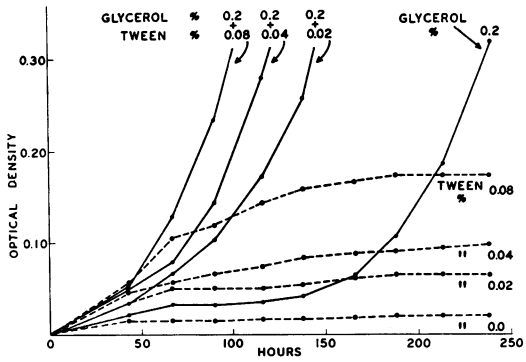

FIG. 1. *Growth of Mycobacterium kansasii in media with and without Tween 80.*

granules. After incubation for 2 hr, the bacteria appeared broader, some showed a central vacuole or zone of greater transparency, and others had vacuoles in the polar regions and an intensely stained band across the middle portion of the cell. After 6 hr of incubation, in addition to the forms described, bacteria with two dumbbell-shaped crossbars, separated by empty spaces, were seen. Sometimes these crossbars were grouped in pairs, suggesting that they had originated from the splitting of one bar along its long axis. After 24 hr, the size of the bacteria often had tripled, and each bacterium had numerous crossbars. After 48 hr, microcolonies had formed, composed of long chains of cross-barred bacteria. By the 7th day, most bacteria were again regular, homogeneously stained rods of normal size. A similar sequence of transformations was observed in cultures in liquid Tween-albumin or liquid oleic acid-albumin medium. On the other hand, in media without fatty acid, none of these changes was observed, and the bacteria always appeared as homogeneously stained rods.

Studies of the quantitative relationship of the morphological changes to the amount of fatty acid added indicated that, within certain limits, the intensity and the duration of the cross-barring phenomenon increased with the amount of fatty acid added. Cultures from media with fatty acid which had gone through the cross-barred stage, and were growing as uniformly stained rods, became cross-barred again after addition of fatty acid. This finding indicated that the cross-barring was not the attribute of a particular growth phase, but depended solely on the presence of fatty acid in the medium.

Staining procedures. Attempts to identify the nature of the cross-bars by selective staining methods gave the following results. The cross-bars could not be stained by the method for staining cell walls or cell septa (Fig. 3a) but

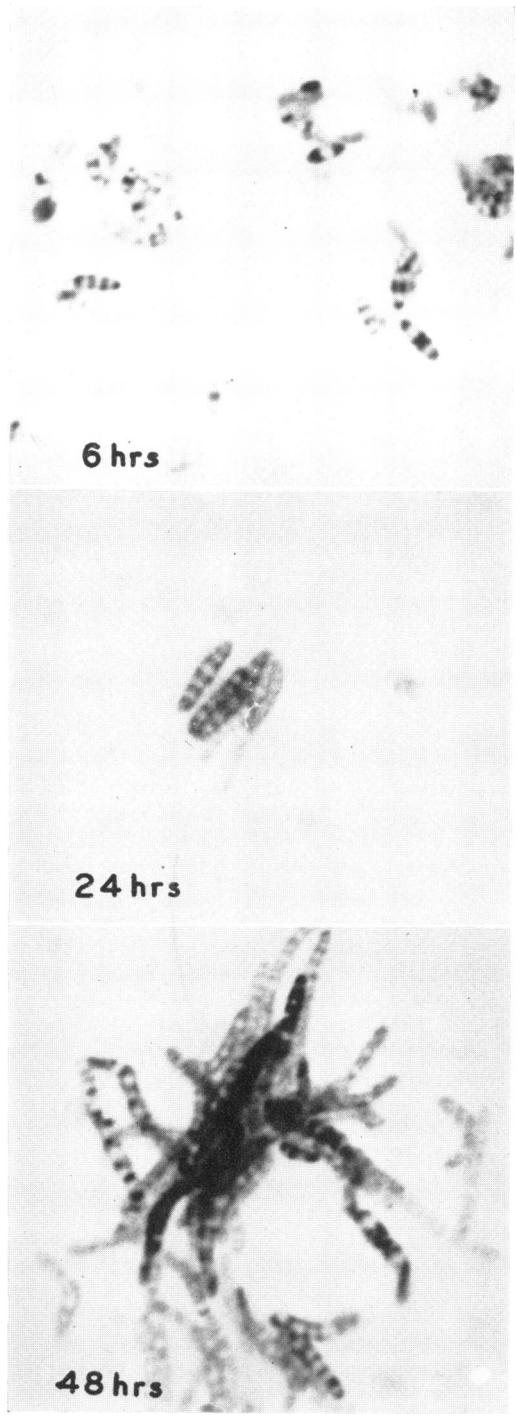

FIG. 2. *Morphological changes of Mycobacterium kansasii during growth on oleic acid-albumin-agar medium. Impression smears made at 6, 24, and 48 hr of incubation at 37 C. Ziehl-Neelsen cold stain.* × *3,000.*

were stained by the Giemsa stain for nuclear chromatin (Fig. 3b). The Feulgen stain for deoxyribonucleic acid did not give clear results. The periodic acid-Schiff stain for carbohydrate gave results similar to those of the acid-fast staining procedure. On staining with Sudan black B, the bacteria appeared as homogeneously stained rods or as chains of large black beads (Fig. 3c). The diameter of these black areas appeared to exceed the cell diameter, suggesting that the dye was bound to the cell surface and not to globules within the cells.

In an attempt to determine more precisely the location of the fat stain in the bacteria, smears stained by Sudan black were restained with the Ziehl stain. The latter stain, however, did not color the Sudan black-stained bacteria, but revealed the presence of other bacteria that had not been stained by Sudan black. By repeating the Ziehl staining procedure, it was finally possible to superimpose carbolfuchsin staining on the Sudan black-stained bacteria. Pictures of such bacteria, before and after restaining, are shown in the center of Fig. 4a and 4b. It can be seen that the beads in the bacteria, stained by Sudan black, correspond to the sites stained later by carbolfuchsin, whereas the unstained interspaces correspond to the vacuoles seen in the Ziehl-stained bacteria. This is most clearly seen at the site marked by an arrow. This result argued against the presence of fat in the vacuoles but did not exclude the presence of fatty acid or some other lipid at this site.

Phase-contrast microscopy. Bacteria from a 20-hr culture in fatty acid-containing medium were fixed with formalin, mounted in water under a cover slip, and examined with a phase microscope. They resembled inflated bags with rather regularly spaced constrictions, and no details of the internal structure could be seen (Fig. 5a). After the cells were dehydrated by successive baths in alcohol of increasing concentrations and the preparation was left for 2 days in absolute alcohol, the bacteria assumed the typically cross-barred form (Fig. 5b). Dehydration by P_2O_2 did not have a similar effect on the morphology of the cells, suggesting that the effect of alcohol was due not so much to its dehydrating as to its lipid solvent properties.

To observe the internal structure of the living bacteria, cells from a Tween-albumin culture were mounted in 32% gelatine under a cover slip and were examined by bright phase-contrast microscopy (Mason and Powelson, 1956). After 4 to 7 hr of incubation, the bacteria were short rods, most often with highly refractile globules at the poles and in the middle of the cell. After 18 hr of incudation, the size of the bacteria was doubled, and the whole body was

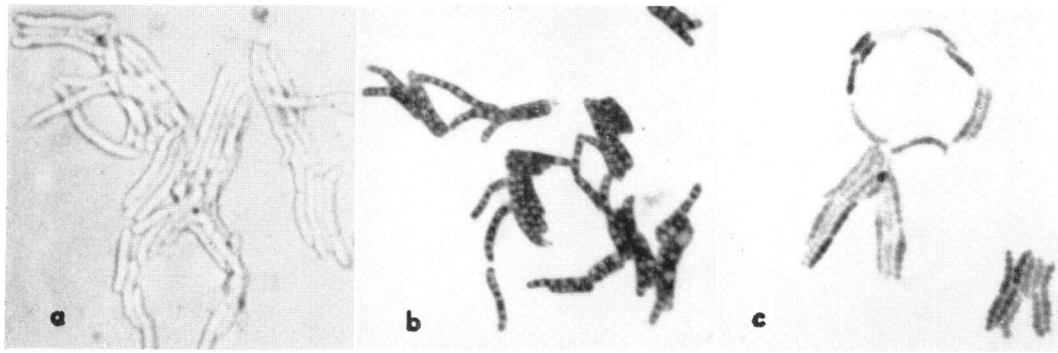

FIG. 3. *Cross-barred forms of Mycobacterium kansasii stained by (a) tannic acid-gentian violet for cell wall and septa, (b) HCl-Giemsa for nuclear chromatin, (c) Sudan black B for fat.* × *3,000.*

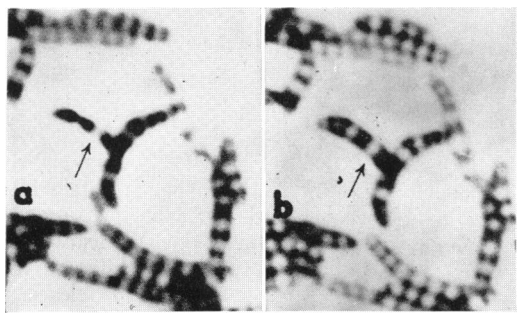

FIG. 4a. *Culture of Mycobacterium kansasii (1 day old) on oleic acid-albumin-agar medium. Smear stained by Sudan black B and by the Ziehl-Neelsen cold stain. Bacteria in center took the fat stain; cross-barred bacteria, at the periphery, took the acid-fast stain.*

FIG. 4b. *Same smear restained with the Ziehl-Neelsen cold stain. The arrows in Fig. 4a and b indicate corresponding site of the same bacterium.*

filled with six or seven large brilliant globules. At a later stage, when the amount of Tween in the medium approached exhaustion, the globules were less bright and distinct. By the 5th day, the bacteria again were small, and their interior was transparent except for some denser areas adjacent to the poles and the lateral cell wall. Similar results were obtained with media containing oleic acid instead of Tween 80. Bacteria from media without fatty acid showed very little internal differentiation.

Effect of fatty acids on the turbidity of bacterial suspensions. To determine whether the change in the structure of the bacteria observed under the influence of fatty acids also caused a change in the turbidity of their suspension, washed bacteria from a fully grown culture were suspended in a culture medium to give it a measurable turbidity. Various sets of this suspension were supplemented with Tween 80, oleic acid, or glycerol, or with Tween and glycerol, and turbidity readings were made at short intervals. The results of such an experiment are represented in Fig. 6.

In the medium without fatty acid and with glycerol as the sole carbon source, the turbidity increased slowly but steadily, apparently proportional to the amount of bacillary growth. In the media with Tween or oleic acid, the turbidity increased almost instantaneously and continued to increase for 2 to 3 hr, at which time it had reached a two- to threefold higher level than initially. From then on, the turbidity remained constant in those cultures in which Tween or oleic acid was the sole carbon source, whereas a further, but slower increase took place in the culture with glycerol as the second carbon source. The fact that the turbidity increased after the addition of fatty acids within a small fraction of the generation time (*unpublished data*), and the fact that this phenomenon took place even in simple bacterial suspensions in phosphate buffer, suggested that this effect was not due to bacterial multiplication. Rather, the effect appeared to result from a change in the optical properties of the bacteria, caused by the rapid uptake of fatty acid from the medium and its accumulation within the cells in the form of discrete droplets.

Volume changes. To determine whether the volume of the bacterial mass was increased after the turbidity increase, the volumes occupied by the bacteria, after incubation for 3 to 4 hr in media with and without fatty acids, were determined by centrifugation and subsequent measurement of the column height of the sediment in Hopkins' tubes. Whether incubated in the presence or absence of fatty acid, no significant differences between bacteria were found, indicating that the turbidity increase in the presence of fatty acid was not due to growth or swelling of the bacteria.

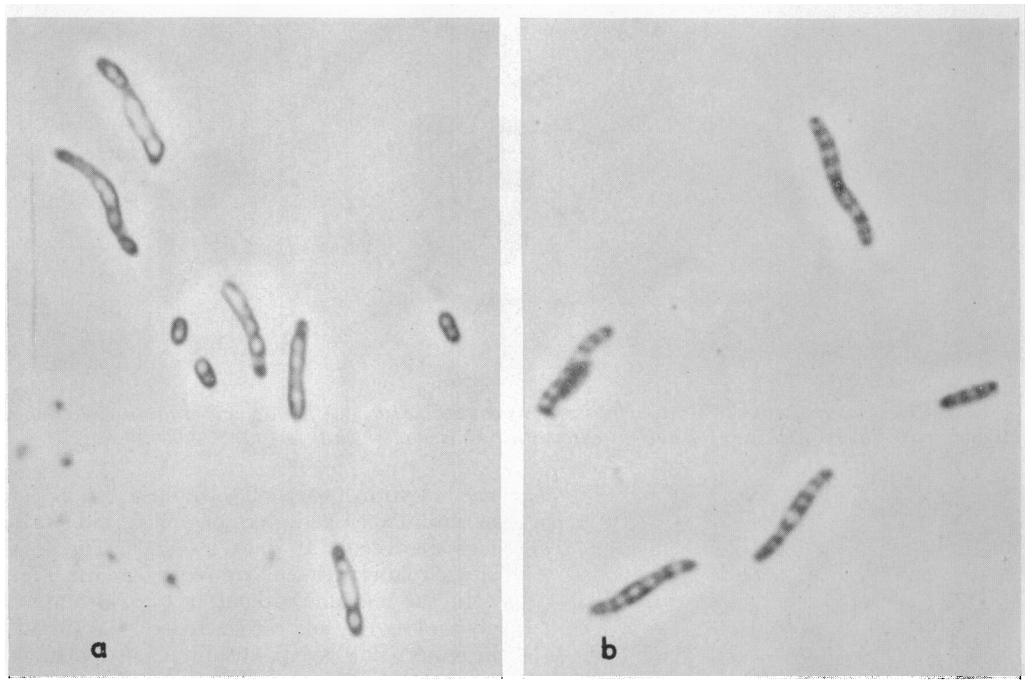

FIG. 5. *Cross-barred forms of Mycobacterium kansasii mounted in water. Phase-contrast. (a) Formalin-fixed bacteria. (b) Formalin-fixed and alcohol-treated bacteria.* × *3,000.*

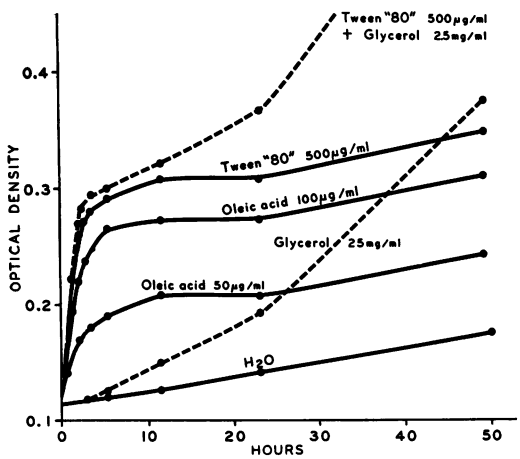

FIG. 6. *Turbidity changes in suspensions of Mycobacterium kansasii during incubation in media with and without fatty acids and with and without glycerol.*

Electron microscopy. For the analysis of the fine structure of the bacteria, ultrathin sections were made and studied in an electron microscope. Figure 7 shows a longitudinal section through a bacterium from a 2-week-old culture made in a medium without fatty acid. It is a rod 0.9 μ long and 0.3 μ wide. A fibrillar network in the central part of the cell corresponds to the nucleus. The cytoplasm contains numerous dense particles of variable size, the largest ones having a diameter of about 300 A. They resemble ribosomes and polyribosomes. In the upper third of the cell, between the nucleus and the cell wall, a laminated structure, called mesosome (Robertson, 1959), is seen. A similar structure is found near the lower pole of the cell.

Figures 8 and 9 show similar sections of the bacteria from a 19-hr-old culture in Tween-albumin medium. The cell shown in Fig. 8 is in the dividing stage. Its length is 2.5 μ and its width, 0.6 μ. The interior contains numerous globules of various sizes, the largest ones occupying nearly the whole space between the opposite walls. The globules contain a homogeneous mass of a density similar to that of the matrix of the cytoplasm. They are not bound by a unit membrane. The spaces between the globules are occupied by a granular cytoplasm and by fibrillar strands of nuclear material (N). The globules in Fig. 9 contain small, dense bodies (P; polyphosphate granules). Mesosomes (M) are seen at the dividing line and near the lower pole of the cell in Fig. 8. Such bodies are also seen in Fig. 9, near the center of one cell and in the cross section of

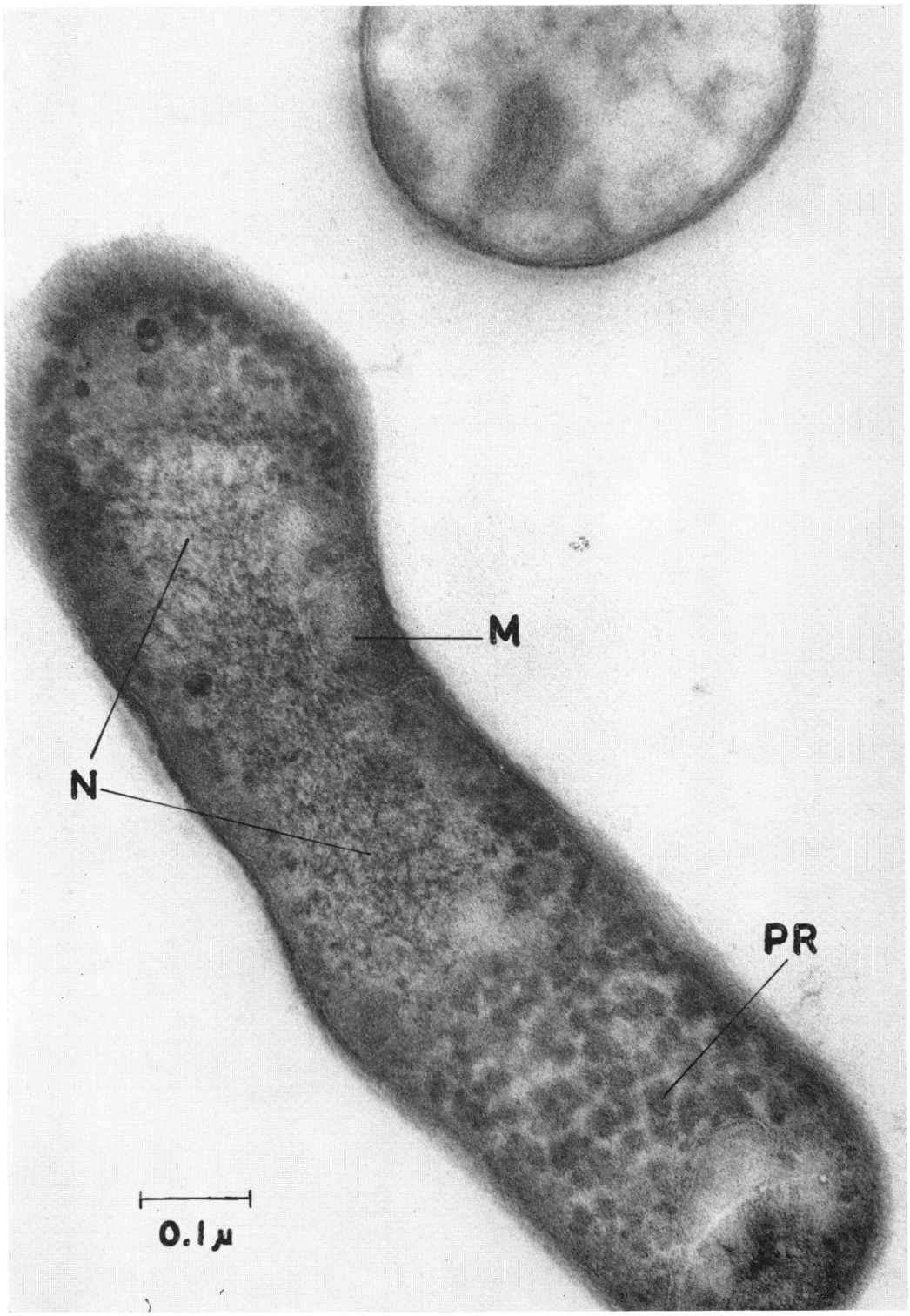

FIG. 7. *Culture of Mycobacterium kansasii (2 weeks old) in medium without fatty acid.* × *170,000. N = nucleus; M = mesosome; PR = polyribosomes.*

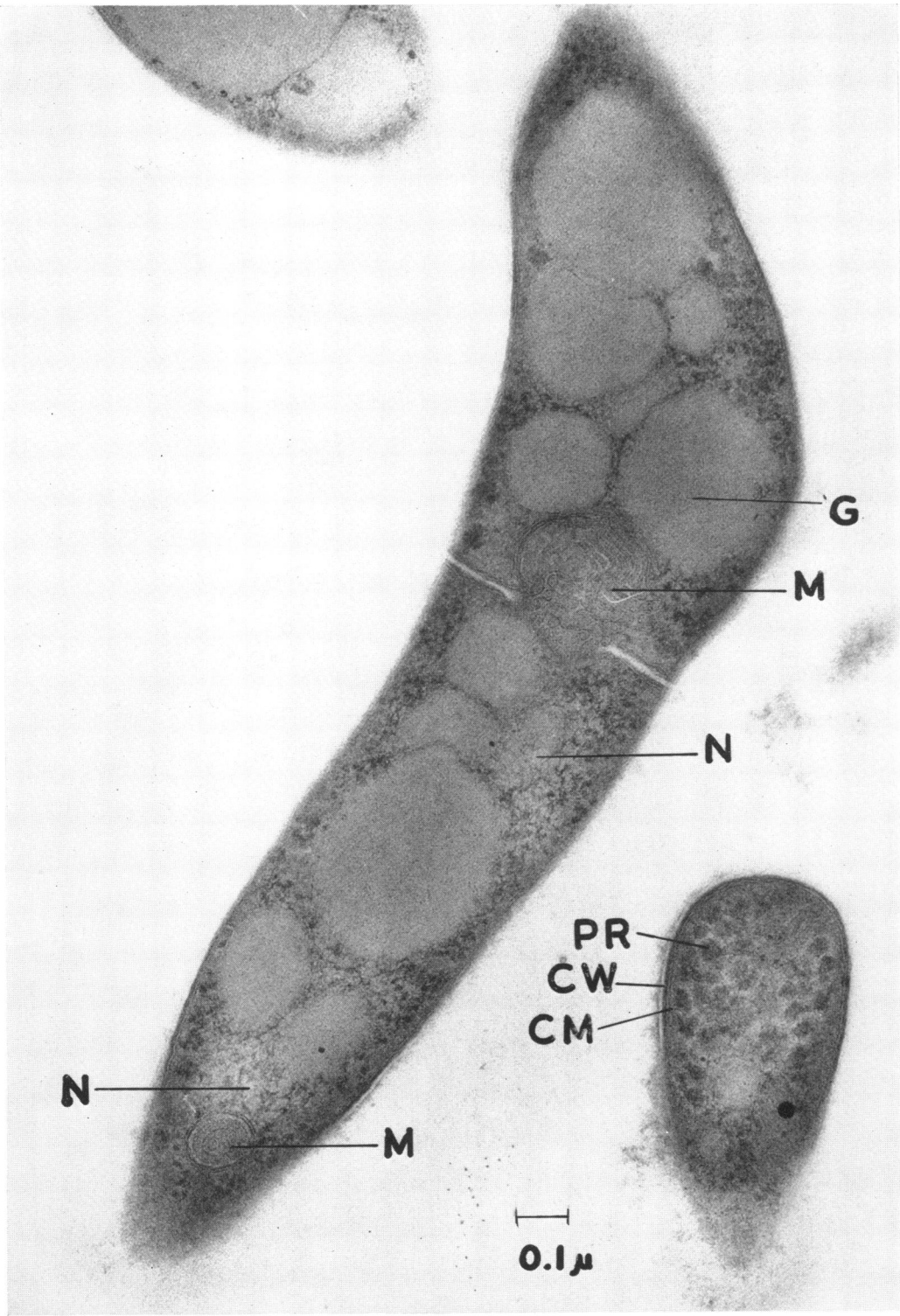

FIG. 8. *Culture of Mycobacterium kansasii (19 hr old) in Tween 80-albumin medium. Bacterium in process of division.* × *80,000. N = nucleus; M = mesosome; PR = polyribosome; G = globule; CW = cell wall; and CM = cytoplasmic membrane.*

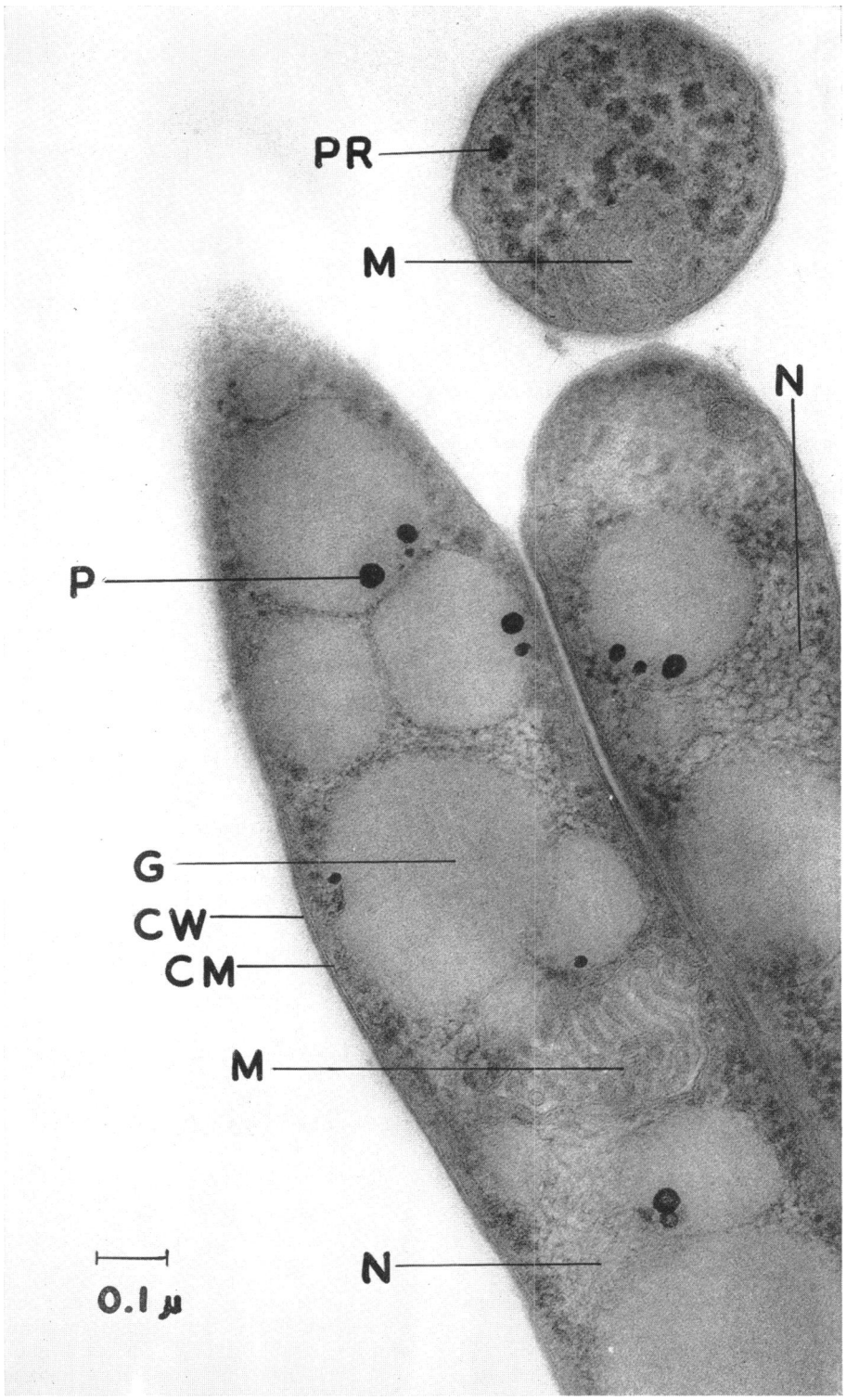

FIG. 9. *Culture of Mycobacterium kansasii (19 hr old) in Tween 80-albumin medium. × 100,000. N = nucleus; M = mesosome; PR = polyribosomes; G = globule; P = polyphosphate granule; CW = cell wall; and CM = cytoplasmic membrane.*

the other. The cell wall and the cytoplasmic membrane can also be seen.

Physiology. To determine whether the described effects on morphology and optical density resulted from a physicochemical or from a metabolic effect on the bacteria, the influence of anaerobiosis and low temperature was investigated. Anaerobic conditions were obtained by use of Thunberg tubes from which the air had been evacuated. The fatty acids were added from the side arm. Under these conditions, no cross-barring and no turbidity increase occurred. Incubation at 4 C also prevented these phenomena.

The effect of enzyme inhibitors on cross-barring and turbidity increase was also studied. KCN caused marked inhibition at a concentration of 0.001 M, and complete inhibition at 0.01 M. Sodium azide was inhibiting at 0.1 M, and 0.01 M iodoacetic acid caused partial inhibition. Sodium arsenate and dinitrophenol had no effect. All these results indicated that the changes produced by the fatty acids were due to an active metabolic process.

Substrate specificity. The addition of synthetic triolein, elaidic acid, lecithin, cephalin, or Tween 20 (sodium monolaurate) induced the same changes in the structure of the bacteria as did oleic acid. Lauric acid, after neutralization with sodium hydroxide, and dissolution in a 5% solution of bovine albumin fraction V, at a concentration of 50 μg/ml of medium, also induced cross-barring and stimulated growth, whereas Triton WR1339 (Winthrop Laboratories, Rensselaer, N.Y.), a phenolic wetting agent, and cholesterol were inactive. Human, bovine, rabbit, and guinea pig sera in a final dilution up to 1:10 also induced cross-barring, suggesting that this effect might be due to their lipid content. Bovine albumin fraction V was inactive.

Differential effect of free and esterified fatty acid in various mycobacterial species. The described morphological changes were observed not only in *M. kansasii*, but also in other mycobacterial species. Two groups of mycobacteria could be distinguished. One group, composed of *M. kansasii*, *M. balnei* (marinum), and certain skotochromogenic mycobacteria, showed cross-barring and the associated turbidity changes in media containing free oleic acid and in media containing Tween 80, an oleic acid ester. The other group, composed of skotochromogenic mycobacteria isolated mainly from cervical lymph node infections in children, and of strains resembling *M. avium*, isolated from pulmonary and lymph node infections in man and animals, showed these changes in media containing free oleic acid, but not in media containing Tween 80.

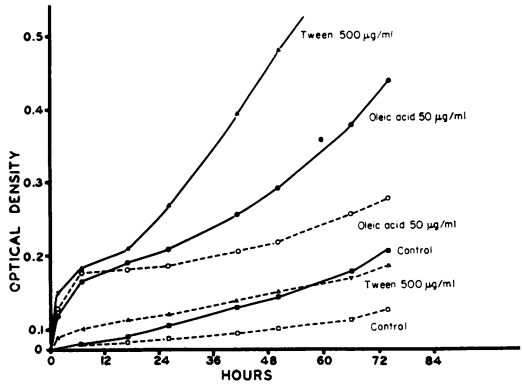

FIG. 10. *Effect of oleic acid and Tween 80 on turbidity of cultures of Mycobacterium kansasii and a skotochromogenic mycobacterium. Solid lines = M. kansasii. Broken lines = skotochromogenic mycobacterium. Symbols:* ● *or* ○ = *oleic acid;* ▲ *or* △ = *Tween 80;* ■ *or* □ = *control.*

The different response of these two groups of strains to free and to esterified oleic acid, as measured by the turbidity changes, is illustrated in Fig. 10. The culture of *M. kansasii*, represented by solid lines, shows a rapid turbidity increase in the presence of free oleic acid as well as of Tween 80, whereas the culture of the second group (a skotochromogen from a cervical lymph node) shows the rapid turbidity increase in the presence of free oleic acid but not in the presence of Tween. The different responses of these two groups of strains to Tween 80 can be explained by the observation of Wayne, Doubek, and Russell (1964), that *M. kansasii* and certain skotochromogenic mycobacteria hydrolyze Tween 80 rapidly, whereas the *M. avium*-like and the skotochromogenic mycobacteria from lymph node infections do not hydrolyze Tween, or hydrolyze it very slowly.

M. tuberculosis and *M. bovis* did not show striking morphological or turbidity changes in media with Tween or oleic acid. Whether or not electron-microscopically visible changes occur in these bacteria as a result of their culture in fatty acid-containing media has not yet been determined.

DISCUSSION

Growth ot *M. kansasii* in a defined medium with glucose or glycerol as the carbon and an ammonium salt as the nitrogen source was preceded by a prolonged lag. The duration of the lag period was reduced when the medium was supplemented with detoxified oleic acid or its ester, Tween 80, which were shown to be utilized by the cells. In such media, the bacteria exhibited

striking morphological changes consisting in the appearance of vacuoles separated by bands of chromatin, the number of which increased with the growth of the cells. The electron microscope revealed, at the sites corresponding to the vacuoles, the presence of single or multiple globules of low opacity. In the phase microscope, the bacteria were seen to contain highly refractile bodies which seemed to correspond to the vacuoles in the stained bacteria. Within a few minutes after the addition of fatty acids, and apparently concomitant with the formation of the globules and refractile bodies, the turbidity of the bacterial suspensions began to rise, and nearly tripled within 3 hr. These processes occurred also in the absence of any nutrient other than fatty acid; anaerobiosis, low temperature, and metabolic inhibitors such as KCN or sodium azide prevented their appearance. These results suggested that the observed phenomena were not a consequence of growth but were due to the active metabolic uptake of fatty acids by the cells and the accumulation of fatty acids in globules. An analogous transient accumulation of storage granules (polyphosphate) has been seen in phosphate-starved bacteria when these were transferred to a phosphate-containing medium (Smith, Wilkinson, and Duguid, 1954; Harold, 1964). The rapid uptake of fatty acids from the medium probably reflects the high capacity of mycobacteria for fatty acid metabolism, providing them with an internal reservoir of a preferred carbon and energy source. The described phenomena of fatty acid uptake were seen in various mycobacterial species. Some showed them in media containing free or esterified fatty acids (Tween 80); others, only in media containing free fatty acids, apparently because these latter strains lack the enzyme for the splitting of fatty acid esters.

The identification of the chemical nature of the lipid accumulated in the cells will be the subject of future studies.

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