OBSERVATIONS ON THE STAINING OF BACILLUS MEGATERIUM WITH TRIPHENYLTETRAZOLIUM

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The reduction of triphenyltetrazolium to its insoluble, red formazan in living tissues was observed by Kuhn and Jerchel (1941). Bielig et al. (1949) demonstrated that tetrazolium reduction by yeasts and bacteria led to a localization of the formazan in red intracellular granules. Similar observations have been made subsequently by Mudd et al. (1951a,b), who interpret the stained granules as bacterial mitochondria. The observations in the present paper cast doubt on the validity of this interpretation.

MATERIALS AND METHODS

Bacillus megaterium, strain KM, was used in all experiments. Cultures were grown in 5 per cent Bacto peptone broth at 30 °C with mechanical agitation. The cells were harvested in the exponential phase of growth, washed by centrifugation, and resuspended in 0.1 m phosphate buffer (pH 7.0) containing 0.25 per cent glucose. A solution of triphenyltetrazolium in water then was added to give a final concentration of 0.005 to 0.05 per cent; over this range, the concentration of tetrazolium did not affect the results. Wet mounts were examined with a Spencer no. 8 phase contrast microscope (dark phase contrast). For continuous microscopic examination wet mounts with a reasonable thickness of suspension between slide and coverslips were used. Continuous observations using thin films of tetrazolium treated bacterial suspensions were found to be unsatisfactory as the sequence of events of tetrazolium reduction within the cells was practically stopped. However, thin films taken at various stages of the process had to be used for photomicroscopy.

Cells were disrupted by treatment for 10 minutes in a Raytheon 9 kc magnetostriction oscillator.

RESULTS

Continuous microscopic observation of a wet mount prepared from a tetrazolium treated bacterial suspension reveals the following sequence of events. The formazan first appears in the form of a considerable number of barely resolvable granules distributed throughout the bacterial cell. These primary granules grow in size and then rapidly coalesce into one or two large secondary granules within each cell. Once formed, about 10 minutes after the tetrazolium has been added, the secondary granules do not change further their shape or size. The whole process can be observed with ordinary illumination, but phase contrast provides clearer differentiation of the formazan granules and accordingly was used for photomicrography. Figure 1 shows unstained cells. Figures 2 and 3 show two stages of formation of primary granules; figure 4, secondary granules. The tetrazolium granules shown in figure 4 correspond closely to the particles described as mitochondria in the photomicrographs of tetrazolium stained B. megaterium published by Mudd et al. (1951b).

The manner in which secondary granules are formed suggests that they do not correspond to the true sites of reduction within the bacterial cells and are probably the product of a purely physical coalescence of smaller formazan particles. This supposition is strengthened by the observation that chemical reduction of a solution of tetrazolium, performed at pH 7.0 by the addition of sodium hydrosulphite, yields a precipitate of formazan which consists of granules virtually identical in size and shape with the secondary granules formed within bacterial cells (cf figures 4 and 5).

That the secondary granules are not identical with the centers of reduction in the cells was proved by the following experiment. A bacterial suspension in the usual phosphate-glucose mixture was divided into two halves, to one of
which tetrazolium was added. When the intensity of staining had reached a maximum, each suspension was sedimented by centrifugation, resuspended in the phosphate-glucose mixture, and subjected to sonic treatment in order to disintegrate the cells. The extracts then were centrifuged

for 15 minutes at 3,000 rpm. This sedimented all the formazan in the extract from the tetrazolium treated cells. The two practically colorless supernates were removed from the sedimented material and mixed with tetrazolium to give a final concentration of 0.05 per cent. Since formazan forms a stable suspension in bacterial extracts, the course of tetrazolium reduction could be followed spectrophotometrically in the Beckman at a wavelength of 550 mμ. In both extracts reduction began immediately and proceeded at a constant rate for a considerable time. The rate of reduction by the extract prepared from cells previously treated with tetrazolium was 77 per cent of the rate of reduction by the extract prepared from untreated cells.

DISCUSSION

The microscopic observations reported in this paper show that vital staining of bacteria with tetrazolium cannot be depended upon to reveal the intracellular localization of centers of reductive activity. The primary sites at which formazan first becomes visible possibly may reflect such localization; but it is quite clear that the large secondary granules are cytological artifacts. This conclusion, derivable from continuous micro-

Figure 1. Unstained cells of Bacillus megaterium.

Figures 2 and 3. Bacillus megaterium cells from a bacterial suspension treated with 0.05 per cent triphenyltetrazolium showing the formation of primary granules.

Figure 4. Later stage showing appearance of secondary granules in Bacillus megaterium treated with 0.05 per cent triphenyltetrazolium.

Figure 5. Formazan particles formed from triphenyltetrazolium by reduction with sodium hydrosulfite at pH 7.0.
scopic observation alone, is confirmed by the demonstration that after rupture of tetrazolium treated cells and removal of the formazan containing granules by centrifugation, the colorless supernatant extract still is able to reduce tetrazolium almost as rapidly as a parallel extract prepared from untreated cells.

In general, indicators like tetrazolium which yield insoluble reaction products may well give misleading results when used to stain intracellular structure in organisms as small as bacteria. The size and appearance of the stained granules are likely to depend largely on the physicochemical behavior of the insoluble product. Therefore, whatever the true nature of the reduction centers in bacteria may be, such indicators are not well suited for investigations of their localization and morphology.

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SUMMARY

It has been shown that the colored granules obtained by staining Bacillus megaterium with triphenyltetrazolium give no certain information regarding reduction centers in this bacterium.

REFERENCES