FURTHER EVIDENCE OF THE EXISTENCE OF MITOCHONDRIA IN BACTERIA[1]

STUART MUDD, ARNOLD F. BRODIE, LOREN C. WINTERSCHEID,[2] PHILIP E. HARTMAN, ERNST H. BEUTNER,[3] AND RUTH A. McLEAN

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

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The granules of mycobacteria, originally described by Robert Koch as spores, have been shown in a preceding communication to be sites of oxidative-reductive enzymatic activity. In respect to several enzymatic reactions, as well as in tinctorial and morphological characteristics, these mycobacterial granules were shown to correspond to mitochondria (Mudd, Winterscheid, DeLamater, and Henderson, 1951). In the present communication granules in several other species of bacteria have been examined by similar criteria. These granules also have been found to correspond in essential characteristics to mitochondria.

METHODS AND MATERIALS

The microorganisms used were: *Escherichia coli*, strain B; *Bacillus megatherium*, a strain originally given us by Dr. C. F. Robinow; *Micrococcus cryophilus*, a large gram-variable coccus isolated in the Department of Agriculture (McLean, Sulzbacher, and Mudd, 1951) and utilized for studies on nuclear mitosis (DeLamater and Woodburn, 1951); and *Saccharomyces cerevisiae*, a strain from baker's yeast and a "cytochromeless" variant, received through the courtesy of Dr. H. M. Davidson, which had been derived as described by Ephrussi and colleagues (Ephrussi, Hottinguer, and Chimenes, 1949; Slonimski and Ephrussi, 1949).

The cells of *E. coli* and *B. megatherium* used had been grown for 3 to 4 hours at 37 C on Morton and Engley (1945) agar slants or in M. and E. broth. The micrococcus was incubated 24 hours at 20 C on similar agar slants or broth prior to use. Both strains of *S. cerevisiae* were incubated 24 hours at 37 C on Sabouraud's agar slants.

The reactions examined for were the reduction of 2,3,5-triphenyltetrazolium and neotetrazolium, probably indicating the action of flavoprotein enzymes, oxidation of the Nadi reagent, indicating cytochrome oxidase, staining with the mitochondrial stain Janus green B, the Baker acid-hematin stain for phospholipid, and the Harman mitochondrial stain. Experimental procedures were followed as in the preceding communication (Mudd, Winterscheid, DeLamater, and Henderson, 1951) with the following modifications:

The reduction of triphenyltetrazolium and neotetrazolium was demonstrated

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[2] National Tuberculosis Association Fellow.

[3] Present address: Sias Laboratories, Brook Hospital, Brookline, Mass.

by adding these compounds to cells suspended in sterile distilled water or the liquid media in which the cells were grown. The final concentration used was 0.005 per cent. Janus green B was simply added to cells suspended in sterile distilled water or buffer in final concentrations from 1:10,000–1:100,000. The cells were allowed to remain in Janus green B for as long as 24 hours. *E. coli*, *M. cryophilus*, and *B. megatherium* were kept in the fast green-aniline solution of Harman's selective mitochondrial stain for varying time intervals up to 1 hour.

EXPERIMENTAL RESULTS

S. cerevisiae. The initial stimulus to the work herein reported was the series of studies by Ephrussi and colleagues upon strains of baker's yeast. They have obtained stable, small-colony strains either by selection of spontaneously occurring variants or by transformation of the wild-type by growth in acriflavine-containing media (Ephrussi, Hottinguer, and Chimenes, 1949). The small-colony variant strains lack the normal capacity of the wild-type for aerobic respiration and are dependent almost exclusively upon anaerobic glycolysis (Slonimski, 1949). The small-colony cells lack cytochromes a and b, cytochrome oxidase, and succinic dehydrogenase activities. Cells of the parent wild-type contain cytoplasmic granules 0.3 to 1 μ in diameter which stain with the Nadi reagent (Slonimski and Ephrussi, 1949). These authors correctly pointed out that the foregoing observations were all in agreement with the hypothesis that the Nadi-positive granules were the counterparts of mitochondria, in which enzymes mediating aerobic respiration are organized in the cells of higher organisms.

Cells of the wild-type of *S. cerevisiae* have been found by one of us (A. F. B.) to contain cytoplasmic granules which stain with triphenyltetrazolium (figures 7 and 8) and neotetrazolium, as well as being the loci of the indophenol blue of a positive Nadi reaction. These granules also give the characteristic sequence of colors with Janus green B. Cells of a small-colony variant (figure 6) showed no granules stainable by the tetrazols, the Nadi reagent, or Janus green B.

E. coli, strain B. Spheroidal granules, for the most part polar in position, have been recognized in electron micrographs of *E. coli* (Claude, 1949; Bielig, Kausche, and Haardick, 1949). Electron pictures of the granules, both within and without phage infected cells of *E. coli*, have been taken by Hillier, Mudd, and Smith and published in Burrows (1949). The polar granules of *E. coli* and related gram-negative rods have recently been shown to reduce tetrazolium (Lederberg, 1948; Bielig, Kausche, and Haardick, 1949; Winkler, 1950) and neotetrazolium (Narahara et al., 1950).

Figure 1 shows a field from a preparation of *E. coli* grown on a salt-glucose medium and lysed with T_2 coliphage. In the upper part of the picture an unlysed but obviously infected cell shows characteristic dark areas in polar and central positions. On each side of the unlysed cell a residual granule may be seen in or on the ghost of a lysed cell. The inserted figure, 1a, shows a phage-infected *E. coli* cell with residual granules near both poles and the center of the cell.

Measurement of these granules liberated from *E. coli* cells lysed by $T2r^+$ bacteriophage, in unpublished electron micrographs by Mudd, Beutner, and

Hartman, shows a range of diameters of 0.2 to 0.4 μ with a mean of approximately 0.25 μ. In other fields what appear to be fragments of these granules are entangled among the ghosts and other cellular debris.

It is to be noted that in the intact cells of *E. coli* these granules are in electron scattering areas near the poles and near the centers of the cells; the dark areas are considerably larger than the granules themselves. We will return to this fact in the discussion.

The formazans of triphenyltetrazolium (figures 4 and 5), neotetrazolium (figure 3), and the indophenol blue of the Nadi reaction in our observations have appeared in these particulate granules at both poles and at one side of the cell centrally. Janus green B stained these same areas and was characteristically reduced in them (figure 2a). Neither the cells nor any portions of them were stained visibly following the application of Baker's acid hematin or Harman's fast-green aniline stains. This failure may well have been due to the small size and inadequate intensity of staining of the granules.

Particulate cellular components of lysed *E. coli* cells have been observed to exhibit, extracellularly, some of the cytochemical properties typical of the intracellular granules previously described. Log phase cultures of *E. coli* in M. and E. broth were infected with T2r bacteriophage and incubated at 37 C until maximum lysis, as determined turbidimetrically, had been reached. Although most observations were performed on mass-culture lysates, the free particles were probably released from the cells by actual bacteriophage infection and not by lysis from without, for free particles were also present in crude lysates when a controlled multiplicity of 8:1 was employed. Following maximum lysis, appropriate concentrations of the various reagents were added to aliquots of the lysates and the reactions studied microscopically. Since the reagents were added only after maximum lysis had been attained, it is unlikely that the action of the granules with the reagents preceded liberation of the granules into the medium.

The appearances of the extracellular particles after the addition of triphenyltetrazolium chloride, neotetrazolium, Nadi reagent, or Janus green B were essentially the same as noted for intracellular granules. A few intact bacteria containing typically stained, well-defined intracellular granules were present in the lysates. The size of the stained free granules and their spherical to ovoid shape were like that of the intracellular granules of the intact cells in the same preparation. The majority of the granules were in irregular clumps or were in pairs and short chains, as if the cell membrane had collapsed about them and mechanically entangled them in the process of lysis. There were, however, many particles which had apparently escaped entrapment and clumping; these particles were found completely free in the medium. In most cases, upon prolonged exposure to the reagents, the extracellular particles did not exert as great a total reaction as did like particles intracellularly, however, qualitatively the reactions were identical. The slightly lessened activity of the extracellular components is possibly due to the deleterious influence of the extracellular broth environment and temperature. Considerable unstained amorphous debris was also present in all lysates.

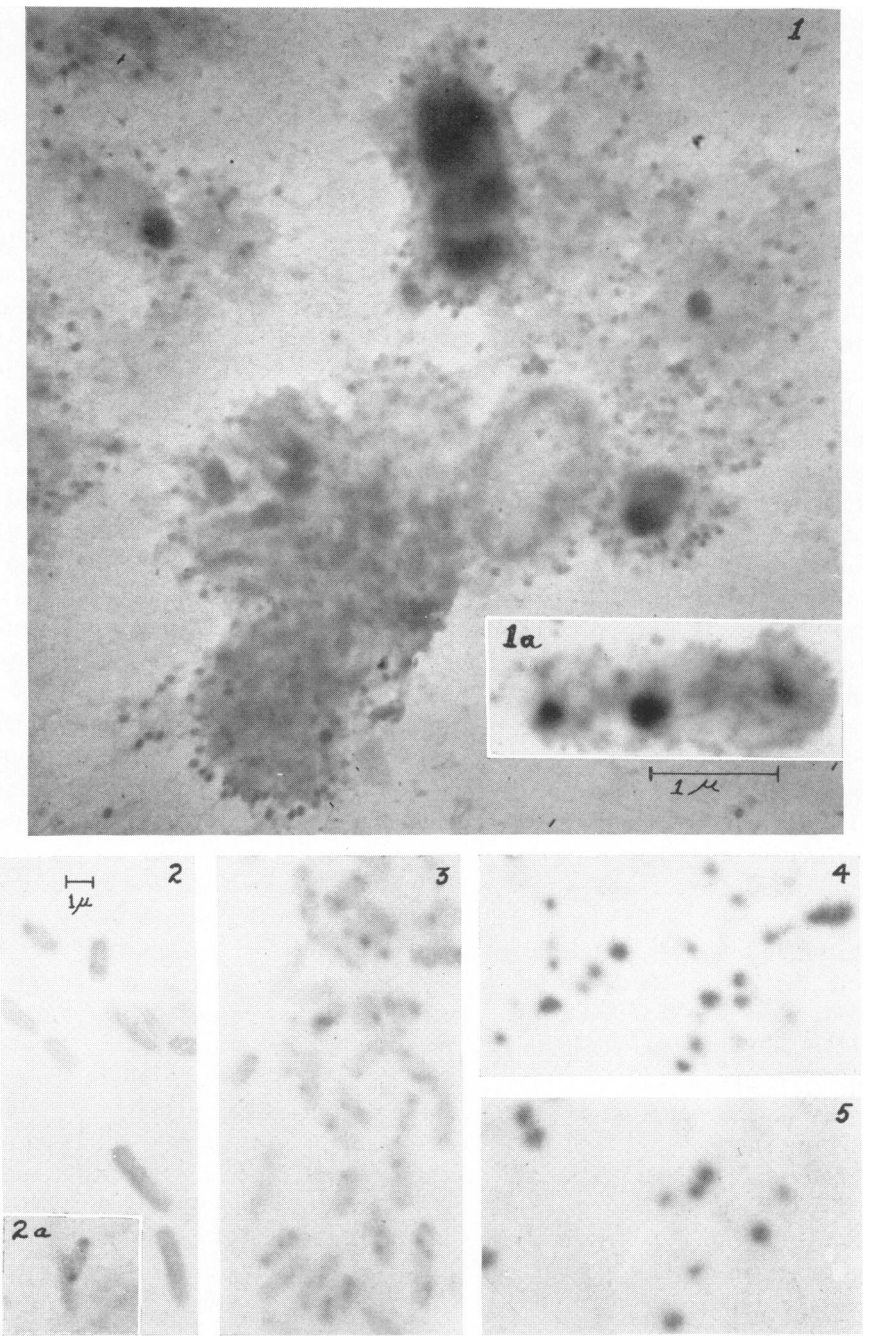

Figure 1. Escherichia coli grown in a salt glucose medium, infected with T_2 bacteriophage for 50 minutes, centrifuged, placed on an agar block, fixed in the vapor of 2 per cent OsO_4 for 1 minute and mounted on a "formvar" film for electron microscopy.

Figure 1a. Escherichia coli grown on collodion on agar (according to the method of

M. cryophilus. The electron scattering by cells of this large coccus, although less than that of fully gram-positive cocci (McLean, Sulzbacher, and Mudd, 1951), is so great as to render the electron microscopic demonstration of internal structure in intact cells difficult or impossible. Electron opaque granules in cells of *M. cryophilus* grown on neotetrazolium agar and probably partially cytolyzed in the subsequent processing are shown in figure 15.

The formazans of triphenyltetrazolium or neotetrazolium first appear in a single discrete granule at the very periphery of the cell within 2 to 4 hours. After longer periods of time the formazans stain more and more granules along the edge of the cell, giving it a beaded appearance which later may look like a continuous ring around the cell (figures 13 and 14). With the Nadi reagent a single peripheral granule was demonstrated. Janus green B stains this granular locus typically, i.e., initially green, changing to red. Following Baker's acid-hematin stain for phospholipids, the cells were light blue with a dark blue-black granule at the periphery and blue-black areas that resemble the nuclear areas. After pyridine extraction the central areas remained blue-black while the light blue of the cells and the dark blue-black peripheral granules were no longer stained. This indicates a concentration of phospholipids in the peripheral granules. No visible staining was produced by Harman's fast green-aniline stain.

B. megatherium. Because of the intense electron scattering of *B. megatherium* we have not yet succeeded in demonstrating internal cytoplasmic granules in electron micrographs. "Electron staining" procedures in starved cells of *B. megatherium* have not yet been attempted.

Light microscopically in many cells the formazans of triphenyltetrazolium and neotetrazolium appeared in discrete polar granules (figures 9 and 10). However, other cells showed round areas whose diameters equalled the width of the cells. They varied in number from 2 to 3, scattered along the central axis of the cell, to so many that they filled the cell completely and were distorted by being packed together. These latter areas were much less intense in color than the polar granules.

Hillier, Knaysi, and Baker, 1948) and infected with T_2 bacteriophage for 15 minutes. The infected cells were dialyzed by floating the collodion support for 60 minutes on cold water and were then fixed with OsO_4 for 1 minute.

Figure 2. Escherichia coli incubated in M. and E. broth at 37 C for 1 hour; a drop of cell suspension was dried on a block of M. and E. agar and an impression made on a cover slip. Cells were counterstained with carbol-fuchsin diluted 1:100 with water for 1 minute. This preparation is a control for comparison with those shown in figures 2a, 3, 4, and 5. Scale on figure 2 applies to figures 2 to 5.

Figure 2a. Escherichia coli, cells grown on M. and E. agar for 4 hours at 37 C and then suspended in distilled water containing 0.005 per cent Janus green B. After remaining in contact with the dye 14 hours many of the cells had changed the dye to the red safranin form of which this cell is an example.

Figure 3. Escherichia coli, same as figure 2 except 0.05 per cent neotetrazolium added to broth at end of hour, then after 5 minutes a drop of cell suspension was removed and processed as in figure 2.

Figures 4 and 5. Escherichia coli, same as figure 2 except triphenyltetrazolium was added and cell remained in broth for 30 minutes. There is no counterstain.

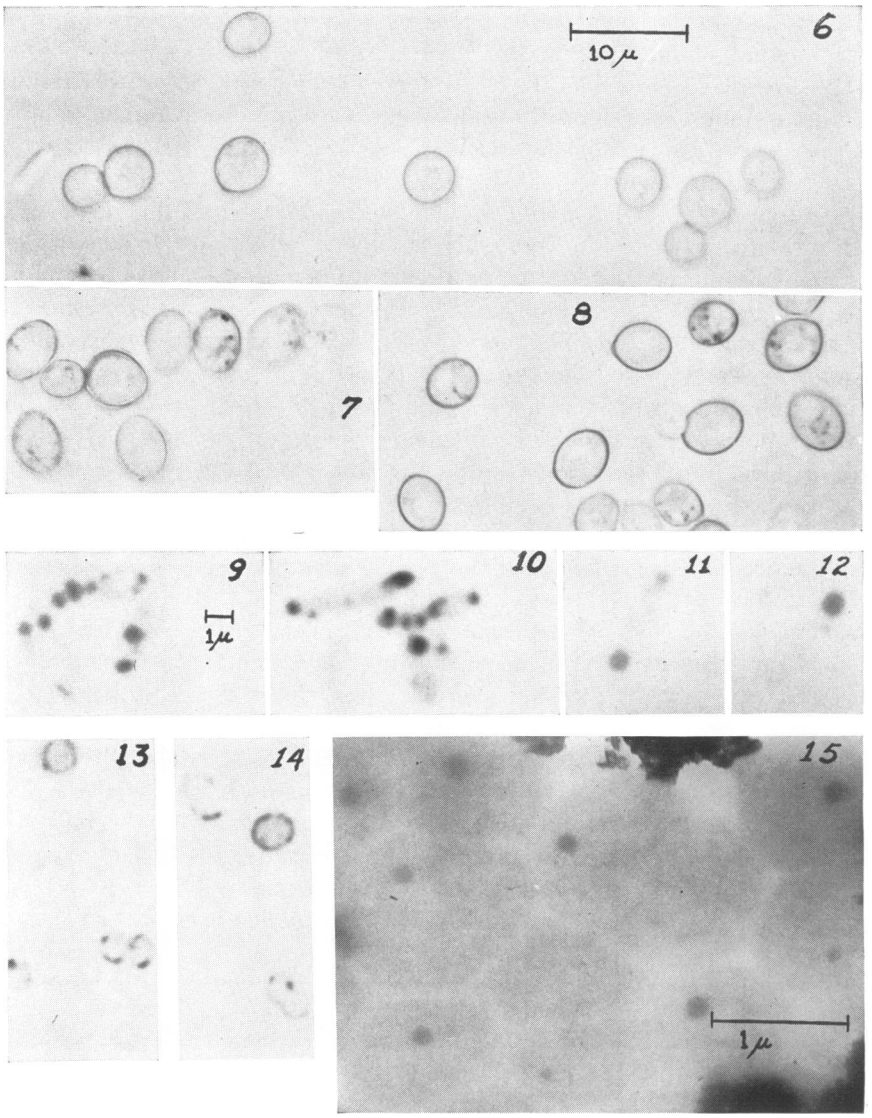

Figure 6. Saccharomyces cerevisiae, small-colony variant grown on Sabouraud's broth 18 hours at 37 C, centrifuged, washed with sterile distilled water, and resuspended in sterile distilled water to which M/15 phosphate buffer pH 7.2 had been added. Triphenyltetrazolium added to make 0.1 per cent, incubated 3 hours at 37 C. Drop of cell suspension sealed on slide under cover slip with paraffin. Scale on figure 6 applies also to figures 7 and 8.

Figures 7 and 8. Saccharomyces cerevisiae (wild-type), same as figure 6 except cells incubated with triphenyltetrazolium 1 hour only.

Figure 9. Bacillus megatherium, an overnight culture was inoculated in M. and E. broth and incubated 1 hour at 37 C. Triphenyltetrazolium added to make 0.05 per cent and cell incubated 30 minutes. Mounts made on cover slips as in figure 2 with no counterstain. Scale on figure 9 applies to figures 9 to 14.

The indophenol blue of a positive Nadi reaction also demonstrated the dark blue polar granules in many cells (figures 11 and 12) and very pale blue round areas in others. Janus green B stained the polar granules characteristically. Baker's acid-hematin stain and Harman's fast green-aniline stain did not give any significant staining of the cells.

DISCUSSION

Cytoplasmic granules in cells of the wild-type of *S. cerevisiae* and in cells of *E. coli*, *M. cryophilus*, and *B. megatherium* are shown to reduce the tetrazols, oxidize the Nadi reagent, and to give the sequence of color changes with Janus green B characteristic of mitochondria. The significance of the tetrazol and Nadi reactions in relation to the respiratory enzyme systems organized in mitochondria has been discussed in a preceding paper (Mudd, Winterscheid, DeLamater, and Henderson, 1951). The mechanism of mitochondrial staining by Janus green B has been under intensive investigation by Lazarow, Cooperstein, and Patterson (1949), Cooperstein and Lazarow (1950), and Lazarow and Cooperstein (1950). In brief the conclusion of these investigators is that:

"The supravital staining of mitochondria within the intact cell appears to be dependent upon: (1) the nonspecific adsorption of Janus green on protein, (2) the rapid reduction of Janus green in the nonmitochondrial portions of the cell, and (3) the localization within the mitochondria of the cytochrome system which slows or prevents the reduction of Janus green at this site."

The extranuclear position of the enzymatically active granules has been ascertained by comparison of preparations stained by the tetrazols, Nadi reagent, and Janus green B on the one hand, with preparations stained by the Feulgen nuclear reaction and by the DeLamater (1951) nuclear procedure on the other (DeLamater and Mudd, 1951; DeLamater and Woodburn, 1951; DeLamater and Hunter, 1951). The mitochondria stain with these indicators of enzymatic activity and the nuclei do not; the nuclear desoxyribonucleic acid is characteristically stained by the Feulgen and DeLamater procedures, the mitochondria are not.

The pattern of contrast in electron micrographs of actively growing cells of *E. coli* has been shown to be due to relatively high electron scattering by the cytoplasmic areas and relatively low electron scattering by the nuclear sites (Robinow

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Figure 10. Bacillus megatherium grown 5½ hours at 37 C on 5 per cent glycerol agar (extract broth base) containing 0.005 per cent neotetrazolium. Counterstained with carbol-fuchsin diluted 1:100 with water for 1 minute.

Figures 11 and 12. Bacillus megatherium, cells grown on M. and E. agar 4 hours at 37 C then suspended in sterile distilled water to which the Nadi reagent had been added. After 34 minutes cells were removed and mounted for observation.

Figures 13 and 14. Micrococcus cryophilus grown on M. and E. agar containing 0.01 per cent neotetrazolium for 5 days at 20 C. Mounted in drop of water on slide and cover slip sealed on with paraffin.

Figure 15. Electron micrograph of *Micrococcus cryophilus* grown on collodion film on surface of M. and E. agar containing 0.005 per cent neotetrazolium at 20 C for 8 hours.

Electron micrographs by Mrs. Jean Minkin, Research Engineer, through the courtesy of the Franklin Institute, Laboratories for Research and Development, Philadelphia.

and Cosslett, 1948; Hillier, Mudd, and Smith, 1949; Mudd and Smith, 1950). It is now clear that the darker cytoplasmic areas are due to the electron-scattering mitochondria and to other electron scattering material (e.g., ribonucleoprotein) surrounding the mitochondria. Since the energy-yielding reactions of aerobic respiration, known to be organized within mitochondria, are coupled with energy-requiring reactions of synthesis, it is an obvious hypothesis that the electron scattering materials around the mitochondria may be the products of synthetic processes made possible by oxidative reactions within the mitochondria.

The fact has already been mentioned that intact mitochondria survive phage infection and lysis of *E. coli*. It is significant in this connection that Cohen and Anderson (1946) found that bacterial multiplication of *E. coli* was stopped by phage infection without gross change in the rate of O_2 consumption or the respiratory quotient.

Specific granules exhibiting cytochrome oxidase, succinoxidase, and other enzymatic activities have been isolated from a stenothermophilic bacterium by Georgi and associates (Militzer, Sonderegger, Tuttle, and Georgi, 1949 and 1950; Georgi, Militzer, Burns, and Heatis, 1951). We do not believe these granules can be identified with the intracellular chromatinic areas staining by the HCl-Giemsa procedure. These granules in the thermophile are interpreted by their discoverers as mitochondria, an interpretation in which we certainly concur.

Electron-scattering granules have been recorded in a considerable variety of microbes. These include: mycobacteria (von Borries and Ruska, 1939; Lembke and Ruska, 1940; Mudd, Polevitzky, and Anderson, 1942; Rosenblatt *et al.*, 1942; Knaysi, Hillier, and Fabricant, 1950; Mudd, Winterscheid, DeLamater, and Henderson, 1951); corynebacteria (Morton and Anderson, 1941; van Iterson, 1947; König and Winkler, 1948); various gram-negative rods (von Borries, Ruska, and Ruska, 1938; Piekarski and Ruska, 1939; Ruska, 1940; Bielig, Kausche, and Haardick, 1949); gram-positive and gram-negative cocci (Frühbrodt and Ruska, 1940; Knaysi and Mudd, 1943); vibrios (Mudd and Anderson, 1942; Mudd, Polevitzky, and Anderson, 1942); various spiral organisms (Mudd, Polevitzky, and Anderson, 1943; Dyar, 1947; Babudieri, 1948); *Spirillum volutans* (König and Winkler, 1950); *Donovania granulomatis* (Rake and Oskay, 1948); pleuro-pneumonialike organisms (Smith, Hillier, and Mudd, 1948); rickettsiae (Plotz, Smadel, Anderson, and Chambers, 1943; Babudieri and Bocciarelli, 1943; Eyer and Ruska, 1944; Ris and Fox, 1949); and *Streptomyces* (Carvajal, 1946). Many of the investigators in question made no attempt to explain the nature and function of the electron-scattering intracellular particles; others interpreted the particles in certain bacteria as nuclei, volutin, etc. So far as we are aware, the possibility that the electron-scattering particles might be mitochondria was not considered in any of the articles cited, excepting possibly that of Bielig, Kausche, and Haardick (1949), and those of Mudd, Winterscheid, DeLamater, and Henderson (1951), and Mudd, Winterscheid, and Brodie (1951). It would be premature to discuss here the validity or invalidity of the various interpretations of the electron scattering particles. We do wish to suggest, however, that interpretations of such organelles at the limits of microscopic visibility, unless based

on the full series of presently available indicator reactions for mitochondria and of improved nuclear staining, may be subject to revision on further investigation. Confirmatory evidence by isolation and enzyme assay of the particles, as by Georgi, Militzer, Burns, and Heotis (1951), would be highly desirable.

SUMMARY

Cytoplasmic granules in cells of the wild-type of *Saccharomyces cerevisiae* and in cells of *Escherichia coli*, *Micrococcus cryophilus*, and *Bacillus megatherium* are shown to give indicator reactions for oxidative-reductive enzymes characteristic of the aerobic respiratory activities of mitochondria. In *E. coli* and in *M. cryophilus*, as previously in mycobacteria, these indicator reactions are shown to be localized in electron-scattering spheroidal to ellipsoidal particles. Such particles are not demonstrated by nuclear staining procedures, nor do the nuclei react to mitochondrial indicators.

Electron-scattering particles morphologically similar to the preceding ones, but variously interpreted, have been demonstrated in a variety of bacteria, spiral forms and rickettsiae. It is suggested that certain identification of the nature and function of such particles will require application of a series of mitochondrial indicator reactions and specific nuclear stains, and should be confirmed by assay of enzymatic activities.

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