CYTOCHEMICAL LOCALIZATION OF DEHYDROGENASE AND DIAPHORASE ACTIVITY IN *BACILLUS MEGATERIUM*

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The site of oxidative activity in the bacterial cytoplasm has remained a controversial topic. The difficulty of localizing functions which are known to require highly integrated enzyme systems may depend on the fact that bacterial cells lack internal cytoplasmic structures common to cells of higher organisms. The bacterial cytoplasm consists of granules, about 20 mμ in diameter, which contain numerous enzymes of the electron transport system of the cell (1) and which were shown to carry out oxidative phosphorylation (2). Attempts of Mudd *et al.* (3) and others to visualize mitochondria-like bodies by means of intravital reduction of tetrazolium compounds to formazan

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pigments provided no definite conclusions, mainly because the association of dye deposits with volutin and transient granules of other origin could not be excluded (4).

The present study deals with a further attempt to demonstrate the sites of electron transport within the bacterial cell by using modern tetrazolium methods capable of intramitochondrial localization of dehydrogenase activity. Using 3-(4,5-dimethylthiazolyl-2) 2,5-diphenyltetrazolium bromide (MTT) and cobalt chelation of the resulting formazan, methods for the demonstration of β-hydroxybutyrate (β-OHB) dehydrogenase, succinic dehydrogenase, and reduced diphosphopyridine nucleotide (DPNH) diaphorase activity (5, 6) were applied to the study of whole cells and protoplasts of *Bacillus megaterium*. In *B. subtilis*, the MTT-formazan produced by the reaction for succinic dehydrogenase was shown to be distributed in the form of a spiral (7).

MATERIALS AND METHODS

Bacillus M, a strain of *B. megaterium* (8), was grown in Gladstone-Fildes broth in shaken cultures at 28° and cells were harvested at 2 hour intervals. After twice washing with 0.9 per cent saline, the cells were resuspended in saline, buffered with 0.05 M tris(hydroxymethyl)aminomethane (tris) at pH 7.2, and the suspensions were adjusted to an optical density of 140 in the Klett-Summerson colorimeter (corresponding to a mean value of 0.176 mg. protein N/ml.). Protoplasts were prepared by adding lysozyme (egg white, Armour) 50 μg./ml. to the suspensions containing 7.5 per cent polyvinylpyrrolidone. For the demonstration of dehydrogenase activity, an equal volume of incubating medium was added to 0.5 ml. suspension of intact cells or protoplasts. The reaction was followed at room temperature with dark phase contrast microscopy. For quantitative assay, incubation was carried out for 30 minutes at 37°. The final reaction mixtures contained MTT 5×10^{-3} M, tris buffer 5×10^{-2} M, $CoCl_2$ 2.4×10^{-3} M, substrate (succinate or β-OHB) 5×10^{-2} M, coenzyme (DPN or DPNH for diaphorase assay) 5×10^{-3} M. The enzyme reactions were determined quantitatively by measurement of enzymatically-formed cobalt-formazan, after extraction with ethyl acetate, at 660 mμ in a Zeiss PMQII spectrophotometer.

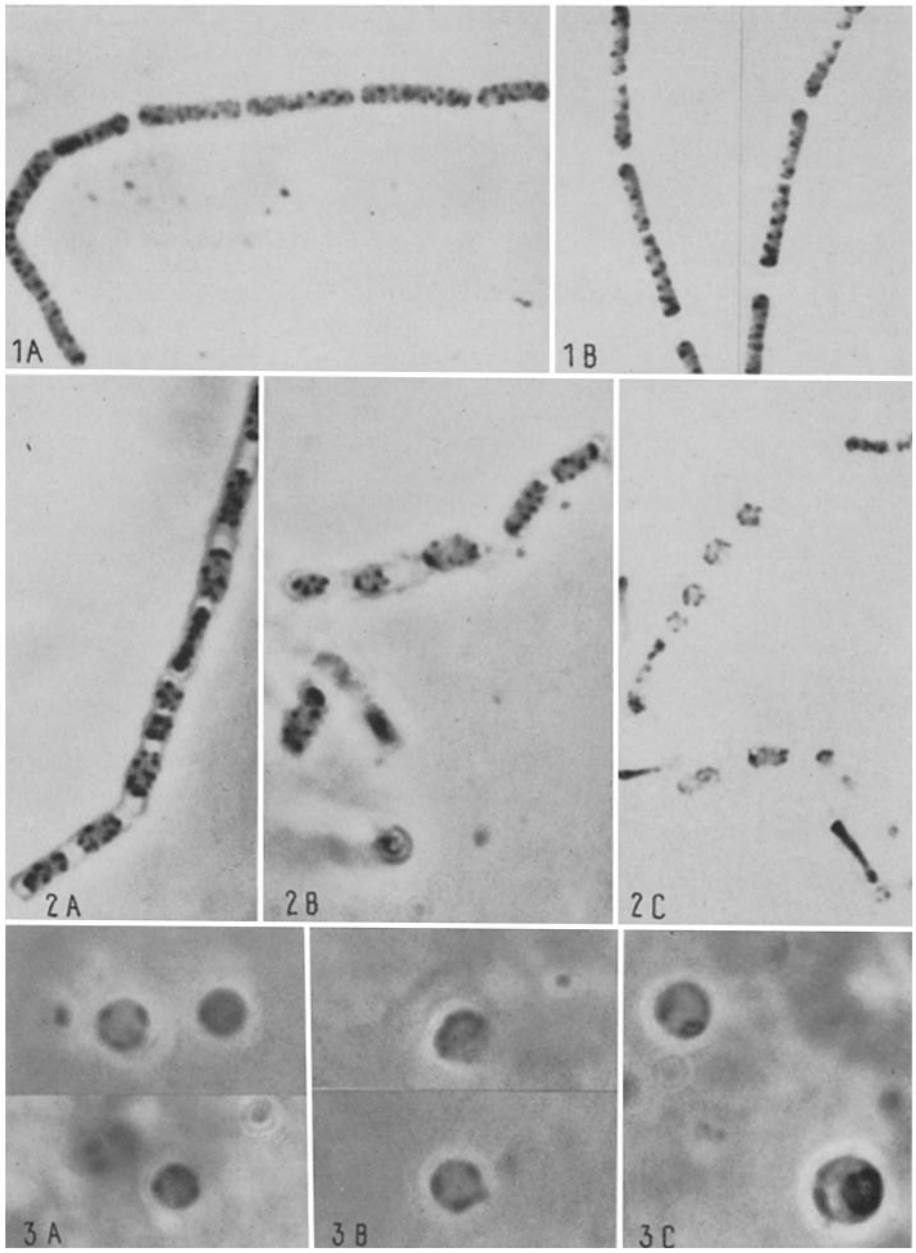
RESULTS AND COMMENT

With all the substrates used, enzymatically-formed dye showed as dots of 0.1 to 0.2 μ diameter at regular intervals within the cytoplasmic region of intact cells (Figs. 1A and B). The formazan deposits may be seen to be disposed as parts of a spiral extending throughout the cell. With the appearance of intracellular lipid granules in older cultures, this structure became irregular, but the lipid granules themselves were not stained. During protoplast formation (Figs. 2A to C), the dye deposits were seen to adhere to the detached cytoplasmic body. When stabilized photoplasts were incubated in the MTT medium at room temperature, the density of the whole protoplasts increased (Fig. 3A). After 10 minutes incubation, dense particles were seen to form a peripheral rim (Fig. 3B), resembling dense areas which may be seen with phase contrast in stabilized suspensions without the addition of tetrazolium (9). After 20 minutes, lysis occurred and dye deposits were seen to be attached in an irregular fashion to empty, ghost-like spheres (Fig. 3C). Cell wall suspensions obtained from mechanically disintegrated cells were devoid of activity.

In contrast to the findings of Davis and Mudd (10), formazan production by intact cells was found to be substrate-dependent and required 15 to 30 minutes incubation in order to be seen in the light microscope, this in spite of the more favourable reduction potential of MTT as compared to the tetrazoles used by these authors (11). Actively growing cells showed higher substrate-dependent activity and lower endogenous activity than cells from older cultures (Fig. 4). The relationship of tetrazolium reductase activity to bacterial growth is the subject of further study.

In additional experiments, the activity of stabilized protoplasts was found to be less than the activity of intact cells and, apparently, diminished as a result of the addition of lysozyme. Lysozyme concentrations too low to induce complete lysis of cell walls (10 μg./ml.) already caused 31 per cent reduction of DPNH-diaphorase activity of the cells. Protoplasts suspended in the MTT-medium containing 100 μg./ml. lysozyme were 38.5 per cent less active than intact cells. This observation is suggestive of an inhibitory effect of lysozyme on respiratory enzymes. Manometric determinations by Weibull (12) have shown identical O_2-uptake of whole cells and protoplasts. Complete lysis of the cells, however, was followed by marked reduction in respiration rate.

The highest rate of tetrazolium reduction was found with succinate as substrate. Table I shows the percentage of activity of DPN-dependent systems in relation to succinic dehydrogenase ac-



FIGURES 1A to 3C

1A. *B. megaterium*, incubation for 30 minutes in β-hydroxy-butyrate medium. 7 hour culture. Magnification × 1600. 1B. 7 hour culture. Incubation in succinic dehydrogenase medium. Magnification × 1300.

2A to C. β-Hydroxybutyrate medium. Stages of protoplast formation. Lysozyme was added after completion of the enzyme reaction. Magnification × 2400 (Figs. 2A and B), × 1600 (Fig. 2C).

3A and B. Protoplasts stabilized for 3 hours in 7.5 per cent polyvinylpyrrolidone. Incubation for 10 minutes in the DPND-medium. 3C. Formazan deposits adhere to the cytoplasmic membrane. 20 minutes incubation in the DPND-medium. Magnification × 2400.

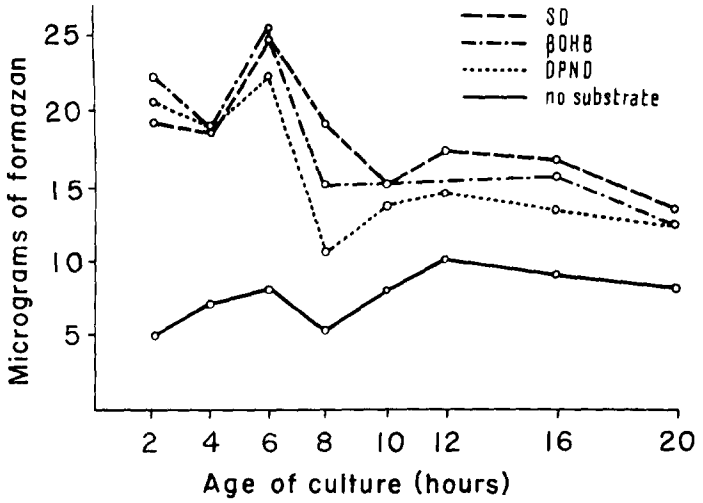


FIGURE 4

Cobalt-formazan production by *B. megaterium* (whole cells). Reactions: succinic dehydrogenase (SD), β-hydroxybutyrate dehydrogenase (βOHB), DPNH-diaphorase (DPND). Points refer to the mean of 3 experiments, each done in duplicate.

tivity in intact cells and protoplasts from 8 hour cultures (mean values of three determinations).

Respiratory inhibitors (amytal, quinacrine-HCL) in concentrations of 5×10^{-2} M did not influence the rate of tetrazolium reduction. Omission of coenzyme in the β-OHB dehydrogenase reaction reduced the activity to 54 per cent. The finding that externally added coenzyme easily gained access to intracellular enzymatic sites is suggestive of lowered permeability of a barrier which has been shown to exist at the surface of the protoplasm (13). This alteration might be due to the action of tetrazolium salt.

The regular arrangement of cobalt-formazan deposits in *B. megaterium* is very similar to that found in isolated mitochondria from various sources (14). Substitution by Nitro-BT (2,2′-di-*p*-nitrophenyl - 5,5′ - diphenyl - 3,3′ - (3,3′ - dimethoxy - 4,4′-biphenylene) ditetrazolium chloride) of the MTT-Co used in the medium gave rise to an analogous formazan pattern, although more contrast was obtained using the metal formazan technique.

The present findings strongly suggest that cytoplasmic constituents of bacterial cells form part of highly organized enzyme units related to particulate mitochondrial fractions, to which enzymic tetrazolium reduction has been demonstrated to be bound (15). The size of the formazan deposits which are resolvable with the light microscope may far exceed the size of the particle responsible for reductase activity. Since identical staining patterns were observed using different substrates, it is conceivable that formazan production in the bacterial cell may depend on particulate multienzyme complexes which, in the case of *Azotobacter vinelandii*, have been shown to be capable of catalyzing the oxidation of both succinate and DPNH (16). Enzyme systems of this sort appear to be spatially organized within the bacterial cytoplasm in a relatively simple manner (17) and it seems unnecessary to postulate the existence of "bacterial mitochondria" (18) to account for the sites where dye reduction occurs. The present cytochemical findings are in accordance with recent studies on subcellular fractions of *B. megaterium* which indicate that both succinate and DPNH oxidase activity is localized in the "ghosts" (*i.e.* cytoplasmic membranes) (19).

TABLE I

Relative Dehydrogenase and Diaphorase Activity of B. megaterium and Protoplasts

Substrate	Whole cells	Protoplasts
	per cent	*per cent*
Succinate	100	38
β-OHButyrate (+ DPN^+)	91	45
DPNH	80	48

The relationship of the formazan pattern observed to a stripe-like localization of reduced triphenyl-tetrazolium or tellurite occurring in several bacterial species (20, 21) remains to be determined. There is a strong possibility that the site of oxidative activity in *B. megaterium* as demonstrated in this paper may be associated with intracytoplasmic membrane systems which have been described recently in various Actinomycetales and eubacteria (*cf.* 22).

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