Localization by Electron Microscopy of Succinic Dehydrogenase in Metabolically Altered Bacillus Cereus During Outgrowth

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LOCALIZATION BY ELECTRON MICROSCOPY OF SUCCINIC DEHYDROGENASE IN METABOLICALLY ALTERED BACILLUS CEREUS DURING OUTGROWTH

by

Mohammed Israr Ali

A dissertation submitted in partial fulfillment of the requirements for the degree of Master of Science in the Loyola University

1968
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INTRODUCTION

Much of our present knowledge of cell biochemistry has been obtained by studies of cell free systems under carefully controlled conditions. The presence of an enzyme or enzyme system in a cell extract indicates only that the cell has the potential to use it. Many factors can influence its activity, including its localization within the cell or cell organelle, availability of substrate, presence of inhibitors or activators, and so on.

The importance of the cellular localization of enzymes within the animal cells was first suggested by Keilin and Hartree (29). A considerable volume of work has since shown that many of the complex enzyme systems involved in oxidative metabolism are localized within the mitochondria in eucaryotic cells and within analogous structures in the procaryotic bacteria. Electron microscopic studies have clearly established that in addition to the limiting plasma membrane there exist in many bacteria an intracellular membrane system (24,31). Thus the bacterial cell is by no means a sacful of enzymes but is in reality a very highly organized system.

The development of electron microscopic techniques has allowed a detailed comparison between structures found in centrifugal fractions of cell extracts and structures visible within the cell. Thus a correlation between biochemical and cytological studies has been established. However, as Marr (64) points out: "the extension of such studies in bacteria is a severe test of the generality of the conclusions based on analysis of larger cells."
The small size of bacteria and their even smaller organelles caused problems both in cytological, as well as in biochemical studies. Until recently artifacts produced by lipid soluble dyes constituted a major problem by dislocating reaction products within the cell, thus casting doubt as to the location of enzymes or other structures within the cell. Difficulties were encountered in biochemical studies, on the other hand, due to the lack of the large, well-defined, structural elements in bacteria, which would serve as guides to the centrifugal separation of homogenates of eucaryotic cells. Thus in bacterial cells, one ends up with 'arbitrary fractions' depending on the specific method of homogenate preparation and centrifugation.

The new tetrazolium dyes, such as tetranitro-blue tetrazolium, have the advantage over the early tetrazolium compounds in that the resulting reduced tetrazolium (formazan) of the new dyes have less affinity for lipids (33,49). Thus, we have now a more reliable method for the localization of enzymes within the structural components of the bacterial cell.

With the relatively recent recognition of organized membranous systems in thin sections of gram positive and gram negative bacteria (6, 7, 17, 24, 37, 38), the question arose as to their function.

These cytoplasmic, membranous structures, first called peripheral bodies, were seen in thin sections of bacteria by Chapman and Hillier in 1953 (3). The presence of other membranous structures were subsequently reported in a variety of bacterial species by various workers (6,7,8,10, 11, 12, 16,24,25, 30, 31, 40,53)
These membranous structures were called mesosomes by Fitz-James (10) and chondiroids by van Iterson (26). The term 'mesosome' is now being used more frequently and this term will be used in this study to describe this membranous organelle.

Attempts to isolate intact mesosomes were not successful and resulted in fractions representing the whole plasma-mesosome system (48); thus, it was suggested that these membranous structures can increase locally the effective area of the plasma membrane (13).

In 1964, Ryter and Jacob (42) first presented evidence that mesosomes connecting the nucleoplasm and plasma membrane in *Bacillus subtilis* were involved in cell division and in the partitioning of DNA between two cells. These observations, based on the analysis of serial section, apparently have been confirmed in *B. subtilis* (44,53) and other bacteria (12). The physical contact of mesosomes with nucleoplasm is more clearly observed in *Lactobacillus plantarum* (60).

In *B. subtilis* the mesosomes are vesicular and are often found attached to the plasma membrane, at the site of cross wall formation during cell division, as well as to other sites (24,26,53).

In 1964-65, van Iterson and Leene found that when vegetative cells of *B. subtilis* were allowed to oxidize succinate in presence of tellurite (27) or tetranitro-blue tetrazolium (TNBT) (53) as electron acceptors, the reduction products were found to be localized in the mesosomes to a greater extent than in the plasma membrane.
In 1965 Sedu and Burde (49) also reported on the localization of the succinic dehydrogenase system in vegetative cells of *B. subtilis*. Using TNBT as the electron acceptor, they found that succinic dehydrogenase activity, as judged by the deposition of TNBT formazan with succinate as the substrate, was located in the mesosomes associated with plasma membrane or the membrane of the newly forming cross wall. TNBT formazan localization was also occasionally observed in the nuclear area, although these workers offered no explanation for the TNBT localization.

An analysis of serial sections of dividing *B. cereus* and *B. megaterium* by Fitz-James (12), showing that mesosomes were attached to nucleoplasm as well as to the newly formed cross wall cytoplasmic membrane, lent further support to the suggested involvement of mesosomes as genome separators and also its involvement in cell division (41,42,45). Mesosomes may also be involved in spore formation (10) and may undergo structural changes when the bacteria are in different metabolic states (5).

Prior to the isolation of mesosomes free from plasma (cytoplasmic) membranes, Salton (47) suggested that those interpretations of differences of function of plasma membrane and mesosome based on the greater intensity of tellurite reduction in the mesosomal region were biased in favor of mesosomes because of their greater membrane surface area (47). He referred to these structures as the plasma-mesosome membrane system (47,48) since up until then all attempts to isolate intact mesosomes had resulted in failure. Furthermore, the evidence for the presence of respiratory enzymes in these structures was lacking. However, Ferrandes et al. (9) recently isolated intact mesosomes of
B. subtilis devoid of plasma membrane and established the presence of almost all the cellular contents of cytochromes in these organelles. Previously the plasma membrane was thought to be the location of all bacterial cytochromes. If these findings are confirmed, it will constitute unquestioned proof that the mesosome is more than a conglomerate of plasma membrane.

A better understanding of the cellular structures and their functions can be obtained by employing synchronous bacterial cultures for such studies, and spores of bacilli provide such a system where natural synchrony can be achieved by rapidly germinating the resting spores (12). "The emergence of a vegetative cell from a dormant bacterial spore represents one of the most direct examples of intracellular differentiation" (51). This transition from dormant spore to a vegetative cell is known to involve two stages: (a) Germination, which is mainly degradative in nature and does not involve protein synthesis; and (b) Outgrowth, which is dependent on anabolic reactions to produce new cellular components.

The tricarboxylic acid cycle has been studied with regard to its role in such metabolic differentiation. It has been suggested that the functions of this cycle could be divided into anabolic, catabolic and amphibolic functions, all of which seem to be under independent control (23).

The anabolic sequence is described as the production of alpha ketoglutarate, i.e. the five carbon skeleton for the biosynthesis of glutamate and the related amino acids of the glutamate family. The catabolic route is regarded as the complete oxidation of acetyl Co A through the cycle with the
subsequent provision of energy. **Amphibolic** TCA cycle functions are those that serve both the anabolic and catabolic requirements of the cell.

The anabolic and catabolic function may respond to different control mechanisms, and these functions may be performed by different sets of enzymes in *B. subtilis* and *B. licheniformis* (Hanson and Cox, 23). However, it is important to note that nutritional conditions may affect the function of the TCA cycle (2, 21). This change in the catabolic function of the TCA cycle was obvious in the experiments of Blumenthal (2) where it was found that germinated *B. cereus* spores that were allowed to undergo outgrowth in dialyzed Casitone medium had a very high respiratory activity as compared to those cells developed to the same stage in undialyzed Casitone. Similar results are being presented in the present study and will be considered later. The catabolic activity of the TCA cycle was assessed by measuring the $^{14}$CO$_2$ released during the metabolism of glucose-6-$^{14}$C. This is based on the fact that C-6 of glucose traversing the Embden-Meyerhof (EM) or another glycolytic pathway does not appear as CO$_2$ until it has passed through an operative, recycling TCA cycle (19, 21).

Since *B. cereus* spores can develop synchronously into vegetative cells with either high or low TCA cycle activity, the present study was initiated to establish the pattern of structural changes and enzyme localization at various stages during the development of the cell. Succinic dehydrogenase, a representative enzyme of the TCA cycle, was selected for study in metabolically altered cells during outgrowth.
MATERIALS AND METHODS

Microorganisms. Bacillus cereus strain T spores which were kept as wet paste at -20 C were used in the present study. These spores were approximately three years old. Also some experiments were performed with freshly prepared spores from this stock culture. The mass production of B. cereus spores is described.

Mass production of spores. Modified G medium has been described previously (1) and was used in this study for the mass production of spores. B. cereus spores, (0.5 g wet weight) after thawing, were suspended in 10 ml of potassium phosphate buffer, pH 7.0, and heat shocked at 65 C for 1 hr. Germination of the heat activated spores was completed within 3 min, after the addition of 50 μmoles of adenosine and 20 μmoles of L-alanine per 0.1 g of spores and aeration at 30 C on a rotary metabolic shaker. These germinated spores were used to inoculate 15 liters of modified G medium in a 16 L borosilicate glass carboy.

The carboy culture was incubated at 30 C, agitated, and aerated by admitting sterile air, employing a pressure pump with a positive pressure of 7-10 lb/sq in, through a submerged inlet. Dow Corning Antifoam (Dow Corning Corp., Midland, Mich.), a silicone spray defoamer, was used to prevent foaming. The effluent air was passed through another carboy containing water and having sterile cotton filters at the inlet and outlet parts of this carboy, before being released to the atmosphere.
Sporulation progress was followed by withdrawing samples from the carboy periodically and examining the cells under phase optics. Under these conditions sporulation proceeded in a uniform fashion and was completed in 28-30 hr. The yield was 45 g of wet, sporangium-encased spores.

The mature spores, still encased, were harvested with a Sharples Super Centrifuge (36,000 x g), the spores being packed onto a sheet of cellophane that lined the centrifuge bowl. Centrifugation of a carboy culture required 40-50 min.

Spores harvested before sporangial autolysis were suspended in cold, sterile, deionized and distilled water. This suspension, containing about 45 g of wet spores in 500 ml water, was then stored at 4 C for 7 days, during which time the vegetative sporangia lysed completely. After 7 days this suspension was further diluted with water to about 2% (w/v) suspension and centrifuged at 16,000 x g for 1 hr at 4 C. The pellet was resuspended in cold deionized distilled water and recentrifuged under the same conditions. The spores were then recomcentrated to about 15 g / 100 ml of deionized distilled H2O and centrifuged at 6,000 x g for 30 min. This wash procedure was repeated about 30 times until the following standards of purity were satisfied.

Standards of purity. The need for clean spore suspensions in any type of investigation was first clearly emphasized by Halvorson and Church (22). The standards commonly employed are given below.

Microscopic: Heat fixed smears, treated with 1% aqueous methylene blue, must be free of stainable materials.
**Enzymatic:** Detectable catalase must be removed. This standard derives from the observation of Lawrence and Halvorson (32) that freshly harvested, intact spores react strongly with hydrogen peroxide. This heat-labile enzyme, presumably a remnant of the vegetative cell absorbed onto the spore surface, can be removed by repeated washings. The catalase activity of washed spores was determined by adding one drop of 30% \( \text{H}_2\text{O}_2 \) to 0.5 ml diluted spore suspension contained in a small test tube. Failure to observe the production of gas bubbles in the test tube constituted a negative test.

**Germinative:** Spores must germinate only in the presence of specific germinating agents. The spores of \( \text{B. cereus} \) strain T, for example, must germinate only with adenosine plus L-alanine (4). In this test, 0.1 g of spore paste suspended in 3 ml of 0.05 M phosphate buffer, pH 7.0, were heat shocked for 1 hr at 65 C in a 50 ml Erlenmeyer flask. This flask was then equilibrated at 30 C and 50 \( \mu \text{moles} \) of adenosine and 20 \( \mu \text{moles} \) of L-alanine were added and incubated at 30 C. The germination of spores was observed after 3 min under phase optics.

All these standards could be met by repeated washings of spores with deionized distilled water. Cleaned spores were stored as a paste in a freezer at -20 C. These spores when needed were thawed at room temperature and used in some of the experiments.

**General experimental conditions.** Generally, 0.1 g of spores (wet weight) was suspended in 3.0 ml of 0.05 M potassium phosphate buffer, pH 7.0, and heated at 65 C for 1 hr. Germination of the heat activated spores was completed within 3 min after the addition of 50 \( \mu \text{moles} \) of adenosine and 20 \( \mu \text{moles} \)
of L-alanine, and aeration at 30 °C on a rotary shaker. Germinated spores would not develop further if allowed to remain in the germination medium (18). These germinated spores were then added to a flask containing 100 ml of growth medium, as described later, and the spores were then incubated on a rotary shaker at 37 °C. Such spores develop synchronously and post-germinative development was followed by removing samples and observing them with phase contrast optics. At the particular stage of development selected, the cells were harvested by centrifugation for 10 min at 10,000 × g at 4 °C. The packed cells were then washed twice in 0.05 M potassium phosphate buffer, pH 7.0, at 4 °C (2). The washed cells at particular post-germinative stage were then resuspended in 5 ml of cold 0.05 M phosphate buffer, pH 7.0, and used as desired.

Isotopic techniques. The relative extent of glucose utilization via hexosemonophosphate (HMP), Embden-Meyerhof (EM) pathways and tricarboxylic acid cycle was estimated on non-growing cells as described previously (2,18,19). A 50 ml Erlenmeyer flask, fitted with a center well containing a removable vial and sealed with a rubber serum stopper, served as the incubation flask. The vial contained 1 ml of ethanolamine-ethylene glycol mono-methyl ether (28) and a filterpaper wick. One ml of 0.1 g / 5 ml suspension of synchronized spores in buffer was placed in the Erlenmeyer flask containing 3.75 ml of potassium phosphate buffer, pH 7.0, and 0.25 ml of appropriately C-1 or C-6-14C labeled glucose resulting in a final volume of 5 ml. Duplicate flasks were used for each experiment, and each flask contained approximately 12.5 μmoles of glucose-14C (33,100 counts / min). A 0.5 ml
Sample of the flask contents was removed for glucose analysis at 0 hr, and a similar sample at the end of the experiment, usually 2 hr or 3 hr. Estimates of the pathways of glucose catabolism were made by the use of a method based on the counts in labelled $^{14}CO_2$ from glucose-$1-{^{14}}C$ and glucose-$6-{^{14}}C$ as described by Blumenthal (2).

The flask was incubated at 30°C on a metabolic shaker. At the desired interval the removable vials were taken out and the entire vial and its contents were added to a 20 ml scintillation vial containing the scintillators in 10 ml of toluene and 1 ml of ethanolamine-ethylene glycol monomethyl ether. The scintillation vial was shaken for 1 min and counted in a Packard Tri-Carb liquid scintillation spectrometer, model 314 E. Corrections were made for the prior removal of samples from the flask for glucose determination.

Electron microscopy. Cells after incubation in growth medium were harvested at the desired stage of postgerminative development, washed twice as described before and suspended in 5 ml of sodium-potassium phosphate buffer, pH 7.2. Generally 1 ml of the above suspension was subjected to experimental conditions, which were essentially the same as described by Seder and Burde (49). The experimental conditions are shown in Table 1. Tetranitro-blue tetrazolium (TNBT) (Sigma Chemical Co., St. Louis, Mo.) was used as electron acceptor to localize the activity of succinic dehydrogenase within the cell. Some preliminary experiments were also performed with thiocarbamylnitro-blue (50) tetrazolium, obtained from Poly Science, Inc., Rydal, Pa. Since TNBT is relatively insoluble in water, it was dissolved in purified N, N-dimethylformamide (reagent) (Baker Chem. Co., Phillipsberg, N.J.). The dimethyl-
formamide (DMF) (25 ml) was first purified by adding 3 g of Nuchar activated charcoal and 6 g of molecular sieve 5 A (Linde Division, Union Carbide, Chicago, Ill.). The suspension was shaken vigorously until an aromatic aroma was detected, after which it was filtered through a hard filter paper (Schleicher and Schuell, 576). Five mg TNBT was dissolved in 0.1 ml filtered dimethylformamide. If a precipitate formed after adding of buffer, the solution was refiltered. No such treatment is needed for TC-NBT which is insoluble in DMF. To each tube containing the incubating medium, as shown in Table 1, 1 ml of cell suspension was added and these tubes were thoroughly mixed and then incubated at 37 C in the dark in either semianaerobic or completely anaerobic conditions. After incubation for 10 min to 15 min the cells were centrifuged at 6,000 X g at 4 C and washed twice in sodium-potassium phosphate buffer, pH 7.4. The cells were then fixed with 1% osmium tetroxide buffered at pH 6.1 as described by Kellenberger et al. (34,43).

The stock buffer solution used in the preparation of Osmium tetroxide fixative contained: sodium Veronal (barbital), 14.7 g; anhydrous sodium acetate, 9.7 g; sodium chloride, 17 g; and distilled deionized water to make 500 ml.

The fixative contained: Stock Veronal buffer, 10 ml; 0.1 N HCl, 14 ml; 1 M CaCl₂, 0.5 ml; osmium tetroxide, 0.5 g; and distilled deionized water, 26 ml.
Table 1. Composition of incubating media for studying activity of succinic dehydrogenase (SDH) in B. cereus spores in elongated stage (of outgrowth)

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Experimental</th>
<th>Dye Control</th>
<th>Competitive Inhibition</th>
<th>Fixation Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M Na$_2$HPO$_4$/KH$_2$PO$_4$ pH 7.2, ml</td>
<td>11.25</td>
<td>11.25</td>
<td>11.25</td>
<td>15.00</td>
</tr>
<tr>
<td>0.8 M Na$_2$ succinate, ml</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
<td>None</td>
</tr>
<tr>
<td>TNBT in Dimethylformamide, mg</td>
<td>15</td>
<td>None</td>
<td>15</td>
<td>None</td>
</tr>
<tr>
<td>or</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC-NBT</td>
<td>15</td>
<td>None</td>
<td>15</td>
<td>None</td>
</tr>
<tr>
<td>Sodium malonate, mg</td>
<td>None</td>
<td>None</td>
<td>500</td>
<td>None</td>
</tr>
</tbody>
</table>
The cells were prefixed with 1 ml of the fixative and centrifuged for 5 min at 6,000 X g. This was the prefixation phase from which a concentrated pellet was obtained. The latter was resuspended in 1-2 ml fixative and 0.1 ml of 2% Tryptone (Case Labs, Chicago, Ill.) solution and left overnight (usually 16 hr) at room temperature. In some of the experiments the Tryptone was omitted without any noticeable difference in results.

After about 16 hr, when fixation was regarded as complete, the suspension was diluted with 8 ml of sodium veronal buffer and centrifuged. The pellet thus obtained was quickly mixed and suspended in about 0.5 ml of warm 2% Noble agar (Difco). The suspension was immediately poured as a drop on microscope glass slide. These manipulations with agar were carried out at about 45 C. After cooling and gelation, each drop was cut into little cubes, with a thin razor blade, which were then dehydrated and embedded as shown in Table 2.

The blocks were sectioned with glass knives using Porter-Blum microtome and thin sections were put on 3 mm grid copper grids 200 mesh size and examined with or without preliminary staining with lead acetate, according to the procedure of Millonig (36), in an RCA-EMU 3 G electron microscope operated at 100 kv.
Table 2. Routine procedure for dehydration and embedding for electron microscopy

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>50% ethyl alcohol</td>
<td>2-5 min</td>
</tr>
<tr>
<td>2.</td>
<td>70% ethyl alcohol</td>
<td>2-5 min</td>
</tr>
<tr>
<td>3.</td>
<td>95% ethyl alcohol</td>
<td>2-5 min</td>
</tr>
<tr>
<td>4.</td>
<td>100% ethyl alcohol: three changes</td>
<td>10 min each</td>
</tr>
<tr>
<td>5.</td>
<td>Propylene oxide: two changes</td>
<td>15 min each</td>
</tr>
<tr>
<td>6.</td>
<td>2/3 Propylene oxide and 1/3 Epon</td>
<td>30 min</td>
</tr>
<tr>
<td>7.</td>
<td>1/2 Propylene oxide and 1/2 Epon</td>
<td>1 hr</td>
</tr>
<tr>
<td>8.</td>
<td>1/3 Propylene oxide and 2/3 Epon</td>
<td>1-2 hr</td>
</tr>
<tr>
<td>9.</td>
<td>Epon mixture</td>
<td>4-6 hr or overnight</td>
</tr>
<tr>
<td>10.</td>
<td>Blocks in capsules with fresh Epon</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Capsules in 45 C oven for 12-16 hr</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Capsules in 60 C oven for 24-48 hr</td>
<td></td>
</tr>
</tbody>
</table>

* Epon is prepared as follows

Epon 812 * | 21.8 ml
Dodecenyl succinic anhydride * | 25.0 ml
Methyl nadic anhydride * | 4.5 ml
DMP-30 ** | 1.5-2%

This should be mixed thoroughly and dessicated in vacuum before use

* These chemicals were obtained from Fischer Scientific, Pittsburgh, Pa.

Propylene oxide from Eastman Organic Chemicals, Rochester, N.Y.
RESULTS

Estimation of glucose catabolic pathways in *B. cereus* spores in elongated stage. Spore suspensions were arrested at the elongated stage (15) of outgrowth after development in either undialyzed or dialyzed Casitone medium. When an estimation of the glucose catabolic pathways was made on the resting spores they typically showed very low yield of $^{14}CO_2$ from glucose-C-$^{14}C$ from cells developed in undialyzed medium, ranging from about 2% to 5% of the total counts added (Table 3). The percent yield of $^{14}CO_2$ from cells developed in the dialyzed Casitone, however, ranged from about 17% to 39%, indicating the existence of a much more active TCA cycle than in cells from undialyzed Casitone. In undialyzed Casitone medium the cells generally utilized more glucose via HMP pathway than cells developed in dialyzed Casitone.

The time to reach elongated stage in the 3 year old spore was about 55 min whereas freshly prepared spores took about 120 min to reach this stage. Results of the isotopic experiments, however, showed no major differences when using either new or old spores. The time for new spores to reach the elongation stage was closer to the time reported by Blumenthal (2).

In the present investigation these metabolically altered spores of *B. cereus* were used for electron microscopic studies on the localization of succinic dehydrogenase in the two cell types during outgrowth.
Table 3. Release of $^{14}$CO$_2$ from glucose-1-$^{14}$C and glucose-6-$^{14}$C by resting spores grown to the elongated stage in dialyzed or undialyzed Casitone medium

<table>
<thead>
<tr>
<th>Casitone Medium</th>
<th>Spore Age</th>
<th>% Yield of $^{14}$CO$_2$ from C-1</th>
<th>% Yield of $^{14}$CO$_2$ from C-6*</th>
<th>HMP$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialyzed</td>
<td>Old</td>
<td>22.1</td>
<td>16.8</td>
<td>5.3</td>
</tr>
<tr>
<td>Undialyzed</td>
<td>Old</td>
<td>21.2</td>
<td>1.9</td>
<td>19.3</td>
</tr>
<tr>
<td>Dialyzed</td>
<td>Old</td>
<td>27.2</td>
<td>20.6</td>
<td>6.6</td>
</tr>
<tr>
<td>Undialyzed</td>
<td>Old</td>
<td>24.8</td>
<td>4.9</td>
<td>19.9</td>
</tr>
<tr>
<td>Dialyzed</td>
<td>Young</td>
<td>40.3</td>
<td>32.0</td>
<td>8.3</td>
</tr>
<tr>
<td>Undialyzed</td>
<td>Young</td>
<td>30.1</td>
<td>4.4</td>
<td>25.7</td>
</tr>
<tr>
<td>Dialyzed</td>
<td>Old</td>
<td>70.0†</td>
<td>39.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Undialyzed</td>
<td>Old</td>
<td>36.0†</td>
<td>4.0</td>
<td>32.0</td>
</tr>
</tbody>
</table>

Three hr experiment in which all glucose was utilized.

+ % Glucose catabolized via the hexosemonophosphate (HMP) pathway as determined by the percentage yield of CO$_2$ from glucose-1- and -6-$^{14}$C.

* A measure of the relative activity of the tricarboxylic acid cycle.
Electron microscopic localization of succinic dehydrogenase. A high percentage of these spores in the late stage of elongation that possessed an active TCA cycle demonstrated the localization of succinic dehydrogenase in the nucleoplasm. No other areas showed localization of the TNBT-formazan. Typical results can be seen in Fig 1 and 2. In Fig. 1, there seems to be some indication of membranous structures, but they are not very clear. In this transverse section, only the upper half of the nucleoplasm shows the TNBT-formazan. In control preparations, where cells with high TCA cycle activity were incubated with succinate, TNBT and malonate, a competitive inhibitor of succinic dehydrogenase, localization of TNBT-formazan was usually not observed, although occasional cells sometimes showed some nuclear localization of TNBT-formazan (Fig. 3 and 4). Similar results were obtained when spores having low TCA cycle were incubated with succinate and TNBT (Fig. 5).

Spores with high TCA cycle activity were allowed to continue their development until they reached the first cell division stage and were then tested in a similar manner. In such cells the TNBT-formazan was located near the newly forming cross wall on somewhat membranous structures, probably mesosomes, as well as in the nucleoplasm (Fig. 6). Also visible are some areas of enzyme activity near the periphery of the cell.

Mesosomes are regarded as the site of respiratory activity (9,16,26,33,37,40) and succinic dehydrogenase is a typical membrane-bound enzyme. Some preliminary experiments were performed in an attempt to partially clear some of the cytoplasmic contents of the spores in the elongated stage of develop-
FIG. 1.B. cereus spore in elongated stage of development having high TCA cycle activity. Reduced TNBT-formazan, indicated by arrow, can be seen in one-half of the nucleoplasm (N). Some membranous structures (M) are visible but not clear. Also showing are the exosporium (EX), outer coat (OC), and inner coat (IC). X 70,000.
FIG. 2. Cross section of *B. cereus* spores in elongated stage with high TCA cycle activity showing localization of reduced TNBT-formazan in nucleoplasm (N), as indicated by arrows. X 40,000.
FIG. 3. *B. cereus* spores in elongated stage having high TCA cycle activity but incubated with malonate, succinate and TNBT. No localization of TNBT-formazan is visible in these cells. X 44,000.
FIG. 4. Cross section of B. cereus spores in elongated stage having high TCA cycle activity, incubated with malonate, succinate and TNBT. No localization of TNBT-formazan is visible in these cells. × 40,000.
FIG. 5. Section of *B. cereus* spore in elongated stage, having low TCA cycle activity showing no deposition of TNBT-formazan in the cell. X 44,000.
FIG. 6. Section of a dividing cell of *B. cereus*, having high TCA cycle activity, incubated with succinate and TNBT. Localization of reduced TNBT-formazan is seen in the nucleoplasm (N) of one cell at left. Localization near the dividing cross wall (S) as well as near the plasma membrane, as indicated by arrows is also seen. X 36,000.
ment in attempt to make mesosomal structures more visible. In such experiments, suspensions of elongated spores were sonicated for 8 min in a Branson sonicator (Micro tip) with intermittent periods of cooling. The sonicated suspension was then subjected to usual experimental conditions. An electron micrograph of such cells is shown in Fig. 7. The nucleoplasm appears partially constricted and membranous structures are now visible in the cell.

In these experiments a new dye, thiocarbamyl nitro blue tetrazolium (TC-NBT) was used in an attempt to detect the localization of succinic dehydrogenase. This dye yields an osmiophilic formazan that is converted to electron dense osmium black in the presence of osmium tetroxide \( \text{OsO}_4 \). This dye, being insoluble in dimethylformamide, was added as such to the buffer in a final concentration of 0.1%. However, this particular tetrazolium did not give satisfactory results under the conditions used, as evidenced by the absence of formazan localization within the cell. In Fig. 8, empty cell can be seen in which the exosporium, inner and outer coats and apparently some membranous structures are visible.

In another attempt to make mesosomes visible, the techniques of Ferrandes et al. (9) were employed. According to these workers, when vegetative cells of \textit{B. subtilis} were incubated in 0.8 M sucrose containing 0.5 mg lysozyme/ml buffered cell suspension for 1 hr, the mesosomes were found to be concentrated at one end of the cell. When such treatment was applied to \textit{B. cereus} spores with high TCA cycle activity in the elongated stage of development, mesosomes were visible near the periphery of the cell (Fig. 9). Such structures were not visible in control cells.
FIG. 7. Section of *B. cereus* spore in elongated stage, having high TCA cycle activity, sonicated for 8 min, and treated with succinate and TC-NBT. No clear cut localization with this new dye is visible, although membranous structures (M) are now visible. X 38,000
FIG. 8. Sonicated cell of *B. cereus* Spore showing relatively empty cell with some membranous structures within the cell and indicated (M) by arrows. Exosporium (EX), inner coat (IC) and outer Coat (OC) are still intact. X 36,000.
FIG. 9. Section of *B. cereus* in elongated stage having high TCA cycle activity, incubated in 0.8 M sucrose and lysozyme for 1 hr. Nucleoplasm (N) is shrunken and some membranous structures (M) at the periphery of cell are now visible. X 60,000.
DISCUSSION

When germinated spores of *B. cereus* were allowed to develop to the swollen or elongated stage in undialyzed Trypticase Soy or Casitone medium there was a block in the TCA cycle and about eight vegetative cell divisions had to be completed before the TCA cycle became fully active (2, 19, 20). However, the TCA cycle activity increased markedly when spores were allowed to develop to the same stage in dialyzed medium (Table 3). The results of the present study thus supported the earlier findings of Blumenthal (2).

Initially, the dialysis experiments gave variable results, indicating that the conditions of dialysis were more critical than originally realized. For example, when a new batch of dialysis tubing was used, the results were not exactly the same as with the old batch of tubing. Similar variation was observed when different batches of Casitone were used. Routine boiling of the dialysis tubing prior to dialysis, a method which apparently equalized the pore sizes in different batches of tubing, lead to uniform results. So did the storage of the dialyzed medium at -20 C.

In view of the fact that the TCA cycle can be divided into both anabolic and catabolic phases, dependent upon different metabolic conditions (2, 21), it is important to note that in the present study the catabolic phase of the TCA cycle has been measured. The C-6 of glucose did not appear as CO₂ until it had passed through a completely operative TCA cycle (19,20) and this was the technique used to estimate the extent to which the TCA cycle functioned. This technique could not estimate the anabolic phase of the
TCA cycle, although such a phase may have been operative in spores grown to the elongated stage in undialyzed Casitone.

Variations in mesosomal structures have been found to occur in *B. subtilis* during growth and sporulation (5). Coleman found that mesosomes composed of double dense line ("DDL") elements were present more frequently in the cells having high respiratory activity and higher cytochrome content, whereas cells with low respiratory activity showed mesosomes with single dense line elements ("SDL"). It has been suggested by Tustanoff et al. (52) that glucose represses the synthesis of lipoprotein in yeast resulting in the inhibition of mitochondrial lipoprotein and subsequently the succinic dehydrogenase and isocitric dehydrogenase, enzymes found in the mitochondrial lipoprotein. A similar inhibition of mesosomal lipoprotein by the repressors of the TCA cycle in Casitone medium could explain the present results. No attempts were made to identify the repressor substances in the present study although they are most likely amino acids and/or peptides.

In the present study, the inability to discern mesosomes clearly in cells with either high or low TCA cycle activity was disappointing. However, it was clear from the electron microscopic study that those cells with low TCA cycle activity do not show any activity or localization of succinic dehydrogenase, although rare cells may do so. In those cells with high TCA cycle activity, on the other hand, the site of succinic dehydrogenase activity was always clear and definitive. In all, 32 experiments were conducted with appropriate controls and more than one block from each variable in each experiment was sectioned and observed in the electron microscope.
A number of functions have been suggested for mesosomes. The reductive sites on these membranous structures along with their appearance suggested their structural and functional relationship to mitochondria (25, 26, 32, 35, 36). The status of the mesosome as a mitochondrial equivalent remained, however, in doubt in view of the fact that no special biochemical features have been demonstrated in these structures which have not been found in the plasma membrane. However, Ferrandes et al. (9) recently showed that isolated and intact mesosomes of B. subtilis contained almost the entire cellular content of cytochromes. Other functions for mesosomes, such as involvement in cell division and partitioning of DNA between daughter cells, have been suggested on the basis of serial sections of B. subtilis (41, 42) and B. cereus and B. megaterium (12). The control function in septation and regulation of DNA replication were first suggested by Jacob et al. (42).

In view of these findings it was hoped that structural differences of these organelles might be more obvious in metabolically differentiated B. cereus spores. It was surprising to find, therefore, that succinic dehydrogenase activity was located in the nucleoplasm with no mesosomal structures visible. These findings were in contrast to previous reports that succinic dehydrogenase activity was associated with the mesosomes of B. subtilis vegetative cells (27, 33, 49). In three-dimensional reconstructions of germinating spores of B. subtilis, the points of attachment were not always found to be localized on the mesosomes during germination. They rather appeared to be joined to the plasma membrane by an intermediate connecting structure much smaller than a mesosome (41). In the present study such
smaller structures were observed in some elongated cells (Figure 1) but their attachment with nucleoplasm was not clear. Apparently this is why Fuhs (14) could not find any connection between mesosome and the nucleoplasm in three-dimensional reconstitutions of B. subtilis cells. Sedar and Burde (49) have, however, noted the localization of TNBT-formazan in the nuclear region of some B. subtilis cells, although they have not offered any explanation for their finding. One obvious, though unlikely explanation for the nuclear localization of succinic dehydrogenase is that the enzyme is indeed located within the nucleoplasm itself instead of being associated with mesosomes not visible in these preparations. It is known that the TCA cycle as well as glycolytic enzymes can occur within the membrane-bound nucleus of animal cells (35). Generally, succinic dehydrogenase is known to be associated with a lipoprotein membrane, although there are exceptions (15, 35, 46).

In the present study, synchronous systems were employed in view of their obvious importance for such studies (8, 12). Fitz-James (12) employed synchronously developing B. cereus and B. megaterium spores and reported that mesosomes did not appear during the first min after germination. However, after 30 min of outgrowth, when DNA replication was well under way, the mesosomes were prominent, usually at the periphery of the cell and were often associated with the nucleoplasm. In the strain employed in the present study, the mesosomal structures were not distinct at any stage of outgrowth, although there were some ill-defined membranous structures visible in the elongated stage, reached at 55 min after germination (Fig. 1).
In spores allowed to proceed to the stage of the first cell division, the TNBT-formazan was found to be located in the nucleoplasm of one of the two daughter cells, as well as near the newly forming septal membrane. TNBT-formazan, though not as intense as in other areas, was also found in localized spots near the plasma membrane (Fig. 6). These results are in accord with the findings of others, that there is a localization of succinic dehydrogenase near the dividing cross wall. Although mesosomes were not clearly seen, it is likely that mesosome-associated respiratory activity is located near the dividing septum and plays an important role in cell division.

In view of the fact that mesosomes were usually connected with the nucleoplasm (8,12, 25, 26, 30, 41,53), attempts have been made to eliminate some of the cytoplasmic contents, so as to make the membranous structures more visible. In such attempts, B. cereus spores in the elongated stage, having high TCA cycle activity were sonicated for 8 min. These cells were found to be intact, as viewed by phase contrast microscopy, although some internal damage was visible in the electron micrographs. The sonicated spores were then subjected to usual experimental conditions, except that the new tetrazolium, thiocarbamynitroblue tetrazolium (TC-NBT) was used (50). This new tetrazolium which has not been previously tested with bacteria did not work well. However, until the new tetrazolium can be shown to work under the same conditions, it is unfair to compare the TNBT and the TC-NBT. The nucleoplasm was constricted in the center and there were some membranous structures visible near the periphery of the sonicated cells. However, no
statement can be made about the localization of succinic dehydrogenase in these membranous structures due to the poor results obtained with TC-NBT.

Another attempt was made to visualize mesosomes in *B. cereus* spores with high TCA cycle activity in the elongated stage. The spores were incubated in 0.8 M sucrose containing lysozyme under conditions similar to those described by Ferrandes et al. (9). They reported that when vegetative *B. subtilis* cells were incubated in 0.8 M sucrose in the presence of lysozyme for 1 hr, the mesosomes were concentrated at one end of the cell. In the present study some membranous structures, probably mesosomes, did become visible at the periphery of the cell after treatment with lysozyme under hypertonic conditions (Fig. 9). Although the spores were not lysed by the lysozyme, the enzyme apparently had an effect on the elongated spores.

These results give some indications for the presence of mesosomes in *B. cereus* spores in the elongated stage of development. The inability to see the mesosomes in normal elongated cells raises the question regarding the adequacy of the fixation technique. The fixation technique employed in these studies preserves nucleoplasm structure better than mesosomal structure. Since hypertonicity in connection with lysozyme and sonication treatment proved to be a more promising approach, it is likely that some further modification of the fixation techniques can be made so as to make mesosomes visible and to provide better contrast for the membranous structures within this organism. Serial sectioning was not done but may be helpful in this regard.
This study confirmed certain results obtained in radioisotope experiments. In both types of studies, the results showed that only those *B. cereus* spores developed in dialyzed Casitone which showed an active catabolic TCA cycle, as compared to the cells which developed to the same stage in undialyzed Casitone.

The status of mesosome as such and its relation to nucleoplasm and the site of TCA cycle activity still remains to be elucidated and probably require modification of fixation as well as other techniques.
SUMMARY

When *B. cereus* strain T were germinated and allowed to develop synchronously to the elongated stage in dialyzed Casitone medium they had high TCA cycle activity in relation to comparable cells developed in the undialyzed medium. TCA cycle activity was estimated in resting cell suspensions by the liberation $^{14}\text{C} \text{CO}_2$ from glucose-$6^{14}\text{C}$ (2).

Electron microscopic studies were initiated in order to study the localization of the TCA cycle enzyme succinic dehydrogenase (SDH) in these two cell types. Tetranitroblue tetrazolium (TNBT) was used as electron acceptor and TNBT-formazan formation within the cells was observed in thin sections with electron microscope as described in Materials and Methods. In cells with high TCA cycle activity, SDH activity was usually present in the nucleoplasm in almost all cells whereas only occasional cells with low TCA cycle activity showed similar formazan localization. With the omission of succinate or the addition of malonate, a competitive inhibitor of SDH, the high TCA cells showed no localization of SDH activity. In spores allowed to develop until the first cell division, TNBT-formazan localized near the dividing septa, some areas of the plasma membrane and the nucleoplasm as well. Mesosomes, considered by some to be the bacterial equivalent of mitochondria, are usually attached to the nucleoplasm and the plasma membrane. However, only occasional mesosomes were seen in sections of this *B. cereus* strain under the conditions used. Thus, no decision can as yet be made regarding the relationship of the mesosome to the high degree of nuclear localization of SDH.
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LITERATURE CITED


