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Central Body of the *Azotobacter* Cyst

LAURA T. PARKER AND M. D. SOCOLOFSKY
Department of Bacteriology, Louisiana State University, Baton Rouge, Louisiana

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ABSTRACT

PARKER, LAURA T. (Louisiana State University, Baton Rouge), AND M. D. SOCOLOFSKY. Central body of the *Azotobacter* cyst. J. Bacteriol. **91**:297–303. 1966.—Sodium citrate was found to effect extensive rupture of cyst coats of *Azotobacter vinelandii*. By filtering a citrate-ruptured cyst suspension through a Millipore microfiber glass prefilter, a preparation of viable central bodies was obtained that contained less than 1% residual cysts and vegetative cells. Electron micrographs showed the central bodies to have a cell wall and cell membrane. Free central bodies germinated into typical vegetative cells. Central bodies exhibited approximately the same resistance to ultraviolet radiation, sonic treatment, and elevated temperatures as did vegetative cells; cysts were much more resistant. Manometric experiments indicated that central bodies and cysts have almost the same oxidative capabilities. Results of resistance studies indicated that the central body is a contracted vegetative cell encased in a protective coat. The cyst coat appears to account for the resistance of the cyst.

The *Azotobacter* cyst is a unique bacterial structure in which a small spherical replica of the vegetative cell, the central body, is encased in a dense, double-layered capsule or coat. The cysts have been shown by Socolofsky and Wyss (5) to to be much more resistant than vegetative cells to the lethal action of ultraviolet light, sonic treatment, and desiccation, although no particularly significant thermal resistance was demonstrated. It was assumed by these investigators that the development of the dense outer coat was responsible for the increased resistance to the deleterious agents, since the acquisition of resistance coincided with the coat formation.

Socolofsky and Wyss (4) indicated that the central bodies could be ejected from cysts which had been ruptured by treatment with the chelating agent, ethylenediaminetetraacetic acid (EDTA), in tris (hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.0). The authors were unable, however, to demonstrate that the released central bodies retained viability. The exposure to these agents apparently caused a weakening of the outer cyst coat, release of the central body, and a resultant decrease in the optical density (OD) of the suspension. Rupture of the cyst coat by a chelating agent was presumably due to removal of metal ions that function in binding the layers of the outer coat together.

Parker and Socolofsky (Bacterial Proc., p. 34, 1965) reported that encysted cells could be ruptured extensively with trisodium citrate as a chelating agent. The present investigation provides an account of procedures involving the use of citrate for the isolation of central bodies in a viable state and presents evidence of their properties in relation to intact cysts and vegetative cells.

MATERIALS AND METHODS

Organism. A. vinelandii (*A. agilis*), ATCC 12837, was the organism used in these studies for the production of cysts and vegetative cells. The cells were grown on the surface of Burk's nitrogen-free salts agar (6) at 33 C with 0.2% *n*-butanol as the carbon source. Cysts were harvested at the end of 7 to 8 days; vegetative cells were collected at 18 hr.

Rupture experiments. Samples (2 ml) of a cyst suspension adjusted to 0.7 OD at 600 mμ were treated with specified amounts of either the disodium salt of EDTA or trisodium citrate. The diluents in which the cells were suspended are indicated in each experiment. The total volume was brought to 3 ml with the appropriate diluent. The change in OD during a 5-min interval was determined at 600 mμ in a spectrophotometer.

Electron microscopy. Preparations of central bodies and encysted cells were collected and fixed with 2% unbuffered $KMnO_4$ for 30 min and were embedded in Maraglas epoxy resin by use of the procedures of Freeman and Spurlock (1). The blocks were sectioned with glass knives on a Porter-Blum microtome. The sections were placed on 400-mesh copper grids, stained with lead hydroxide, and observed in an RCA EMU-3F electron microscope.

Resistance experiments. The techniques of Soc-

olofsky and Wyss (5) were utilized to determine the sensitivity of the cysts, central bodies, and vegetative cells to ultraviolet radiation, temperature, and desiccation.

Sonic treatments were performed by subjecting the cells to the irradiation produced by a Branson model LS-75 sonifier at maximal power settings. Samples were removed at selected intervals for viability determinations. Cell suspensions were maintained at a temperature below 20 C during sonic treatment.

Manometric measurements. Respiration studies were conducted in a Warburg respirometer at 30 C with air as the gas phase. Suspensions of cysts, central bodies, and vegetative cells were washed twice with Burk's nitrogen-free salts solution in an attempt to neutralize residual chelate action, and were then suspended in Burk's salts solution (*p*H 7.8). A quantity of 500 μmoles of glucose was used as the substrate in the flasks used for exogenous respiration studies. Viable counts were made of the cell samples prior to the respiration studies, and oxygen uptake was expressed on the basis of 10^8 cells per milliliter.

RESULTS

Rupture of cysts with EDTA and citrate. The results of an experiment to determine the ability of both citrate and EDTA to reduce the OD of a cyst suspension in 0.05 M Tris buffer (*p*H 8.0) are presented in Fig. 1. The period of exposure was 5 min. The standard rupture system, involving EDTA, is observed to cause a marked reduction in OD with the addition of 0.2 to 0.5 mg of EDTA per ml of cell suspension. Exposure of encysted cells to citrate also effected a pronounced decrease in OD, but a much greater concentration of the chelating agent was required.

Effect of diluents on cyst rupture and viability. The effect of various diluents on the extent of rupture and loss in viability of citrate- and EDTA-treated preparations is presented in Table 1. It is apparent that the central bodies ejected from cysts ruptured with citrate exhibit a high degree of viability when prepared in either Tris or Veronal buffers or in distilled water. EDTA treatment, however, when conducted in either Tris or Veronal buffers, causes a marked reduction in viability. The small loss of viability in EDTA-treated preparations in distilled water is apparently due to the lack of rupture of the cysts under these conditions.

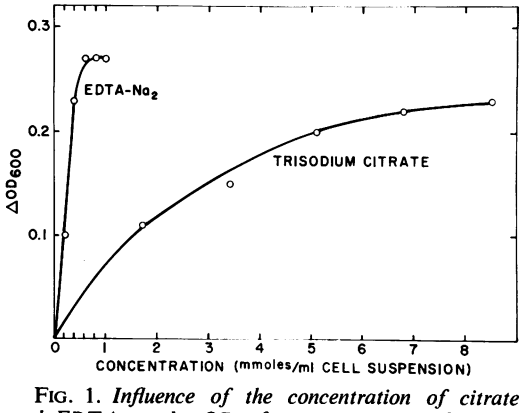

FIG. 1. *Influence of the concentration of citrate and EDTA on the OD of cyst suspensions during a 5-min interval. Cysts were suspended in 0.05 M Tris buffer (pH 8.0).*

TABLE 1. *Effect of several diluents on citrate- and EDTA-treated cysts*

Diluent	Citrate*		EDTA†	
	$\Delta OD_{600m\mu}$	Viability	$\Delta OD_{600m\mu}$	Viability
		%		%
Tris (*p*H 8.0; 0.05 M)	0.20	81	0.24	0.6
Veronal-HCl (*p*H 8.0; 0.05 M)	0.18	87	0.21	18
Distilled water	0.22	94	0.01	98

* Citrate concentration was 2.2 mg/ml.
† EDTA concentration was 0.333 mg/ml.

Action of citrate on germinating cells. Since rupture with citrate appeared to have little effect on the viability of mature cysts, it was of interest to know whether germinating cysts could survive comparable treatment with the chelating agent. Cultures of cysts were washed, suspended in distilled water, and exposed to three different concentrations of citrate (0.5, 0.9, and 1.5 mg/ml) after periods of germination of 3, 6, 9, and 12 hr. In all instances, over 90% of the treated cells exhibited viability, indicating that the citrate was nontoxic to cells at any stage of germination. The sensitivity to rupture by citrate appeared to decrease appreciably, however, prior to the appearance of vegetative cells.

Preparation of central bodies. A method for separating the free central bodies from unruptured cysts and residual vegetative cells was an essential phase of this investigation. Initial attempts with density gradient and differential centrifugation of ruptured cyst suspensions indicated that these techniques would not produce the desired results.

Filtration of a preparation of treated cysts through a Millipore microfiber glass prefilter appeared to separate effectively the central bodies with few residual cysts or vegetative cells. Microscopic observation revealed that a preparation could be obtained that consisted of more than 99% central bodies. Not more than 20 ml could be passed through each prefilter, since filtration of

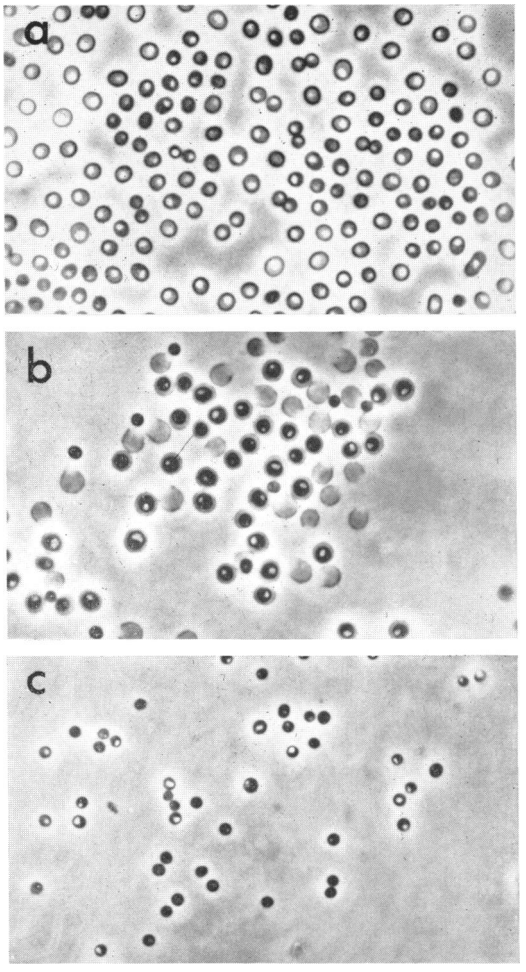

FIG. 2. *Appearance of untreated (a) and citrate-treated (b) cyst suspensions and purified central bodies (c). The concentration of trisodium citrate in the treated preparation was 1.715 mg/ml of cell suspension. Central bodies were purified by passage through a Millipore microfiber glass prefilter and concentrated by centrifugation. In the treated preparation, the exine appears as a halo around the central body of intact cysts. After the central body is released, the exine is observed as a "horseshoe-shaped" structure. The central bodies in the ruptured preparation are identical to those in the purified preparation.*

larger quantities resulted in central body preparations with higher than 1% residual cysts.

To insure that the filtration technique was not merely concentrating small vegetative cells that may be present in an encysted culture, an untreated cyst preparation was filtered. Viable counts of the culture before and after filtration indicated that about 0.02% of the total number of cysts passed through the filter. When ruptured cysts were filtered, approximately 20% of the total number of viable cells were found in the filtrate. Close microscopic observation of the central bodies passing through the filter and those found free in the ruptured suspension revealed that a representative population was present in the purified preparation.

Viability of central bodies. Comparison of direct counts made with a Petroff-Hausser counting chamber and viable counts obtained with standard plating techniques showed that cells in the purified central body preparations exhibited 70 to 90% viability. Occasionally, a population of central bodies was prepared which consisted of less than 70% viable cells. Experiments conducted

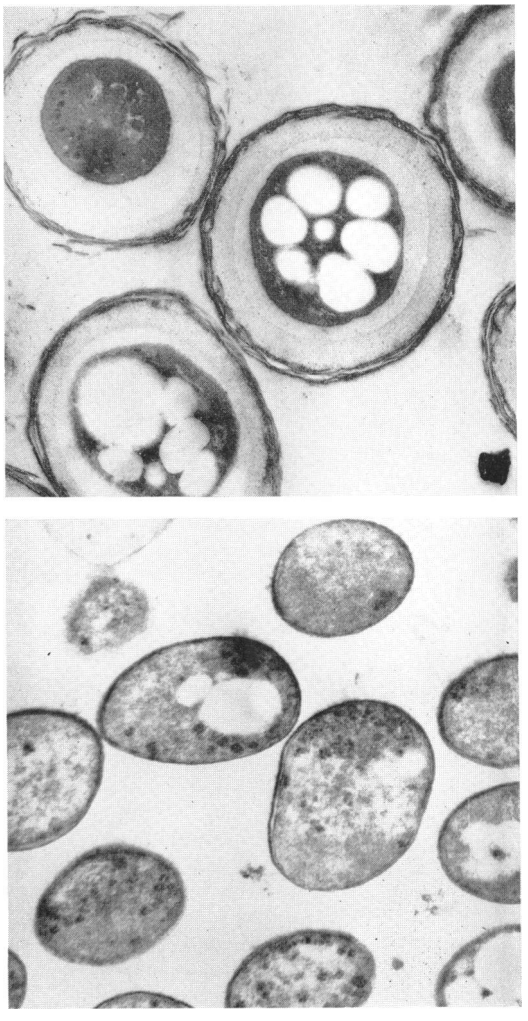

FIG. 3. *Electron micrographs of thin sections of cysts and purified central bodies. Cell walls and cell membranes are evident in the central bodies.*

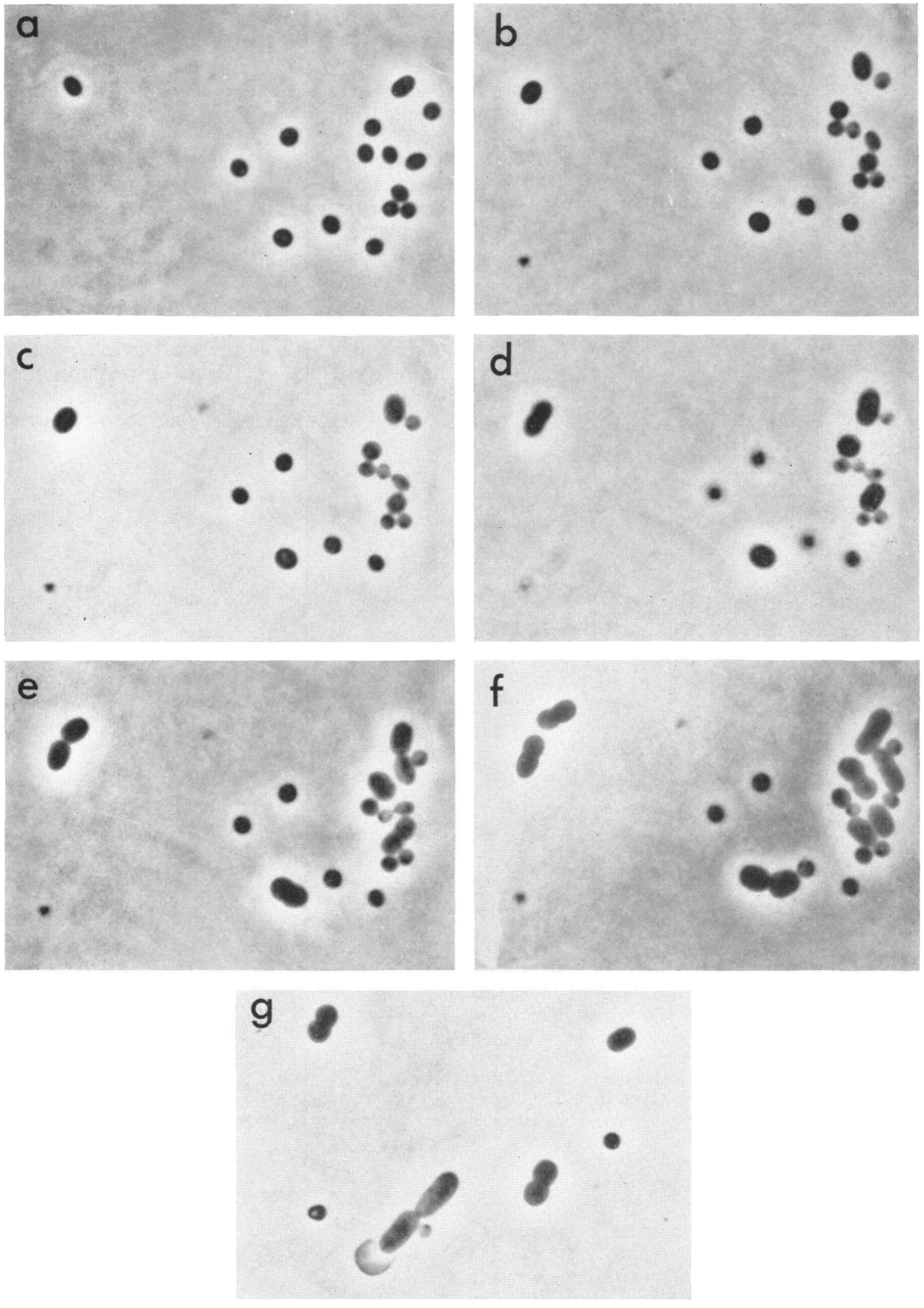

FIG. 4. *Slide culture of purified central bodies of Azotobacter vinelandii cysts viewed with a dark-contrast phase microscope. The central bodies appear to undergo a progressive increase in size until they assume the typical shape of the vegetative cell. The micrographs were taken at the following time periods: (a) 0 hr; (b) 5 hr; (c) 7 hr; (d) 8.5 hr; (e) 10 hr; (f) 12 hr. Figure 3g is a field including a cyst and several central bodies that have germinated. The exine is seen to remain as a "horseshoe" structure after cyst germination. No such structure is seen after central body germination.*

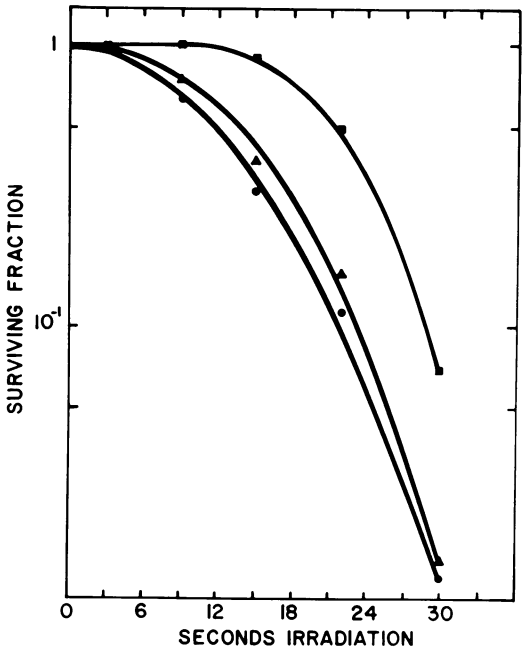

FIG. 5. *Effect of ultraviolet radiation on the viability of central bodies, cysts, and vegetative cells. Symbols:* ■ = *cysts;* ● = *central bodies;* ▲ = *vegetative cells.*

with such preparations were not included in the final analysis of experimental data.

Appearance of central bodies. Figure 2 illustrates the appearance of a central body preparation as contrasted to untreated and ruptured cysts when viewed with a dark-contrast phase microscope. The structures of the cyst are not discernible in the untreated cyst preparation because of the refractive polysaccharide slime layer and thick coat. In the citrate-treated preparation, the refractive mass has been altered to permit observation of the central body, and the exine remains as a "horseshoe-shaped" structure. The free central bodies in the ruptured preparation appear identical in size and morphology to those in the filtered preparation.

Electron micrographs contrasting intact cysts and central bodies (Fig. 3) demonstrate that the central bodies are devoid of the thick cyst coat typical of the *Azotobacter* cyst. The central bodies are observed to possess a cell wall, which indicates that they are not protoplasts.

Germination of central bodies observed by the slide culture technique indicated that a gradual increase in the size of the central body precedes the formation of a vegetative cell (Fig. 4). The cental body undergoes a simple increase in size until it assumes the rod shape typical of the vegetative cell. After the transition to the vegetative state, the cell is able to reproduce itself in the normal manner.

Resistance of central bodies. The ability to obtain purified preparations of central bodies allowed their comparison with cysts and vegetative cells under the conditions of ultraviolet irradiation, sonic treatment, elevated temperature, and desiccation.

The resistance of central bodies to the effects of ultraviolet radiation is presented in Fig. 5. Their sensitivity to the lethal effects of such treatment almost paralleled that of vegetative cells. The cysts were much more resistant to irradiation. A 15-sec exposure reduced the viability of central body and vegetative cell preparations to about 30% of the original, but the cysts were still almost 90% viable.

The results presented in Fig. 6 illustrate the effect of sonic treatment on central body, cyst, and vegetative cell preparations. The central bodies failed to exhibit any more resistance to this treatment than young vegetative cells. A 5-min period of sonic treatment reduced the viability of vegetative cell and central body preparations to less than 1%; 70% of the cells of the cyst suspension survived this treatment for a period of 20 min. The microscopic appearance of the cysts was unchanged after a 20-min sonic treatment.

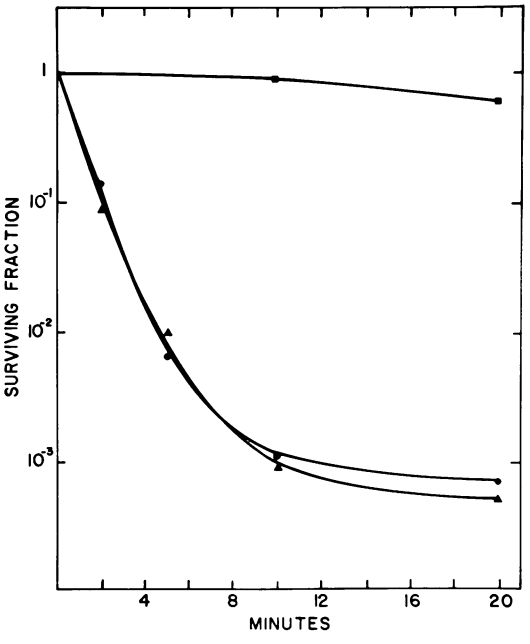

FIG. 6. *Response of central bodies, cysts, and vegetative cells to ultrasonic treatment. Symbols:* ■ = *cysts;* ● = *central bodies;* ▲ = *vegetative cells.*

Neither cysts, central bodies, nor vegetative cells appear to have much resistance to a 15-min exposure to elevated temperatures (Fig. 7). Vegetative cells were least resistant, being inactivated quickly as temperatures approached 48 C. Cysts were able to withstand temperatures as high as 52 C; they were rapidly killed at higher temperatures. The critical temperature for the central bodies seemed to fall between that of cysts and vegetative cells. At 50 C, when almost 100% of the cells of a cyst suspension were viable, only 50% of the central bodies still exhibited viability. This temperature was lethal to almost 95% of a young vegetative cell suspension.

Attempts to demonstrate the sensitivity of central bodies to conditions of desiccation by the membrane impingement method of Socolofsky and Wyss (5) were unsuccessful. In this procedure, the cells are impinged on a membrane filter, exposed to desiccation, and the residual viability is determined after removal of the cells from the membrane by washing action. Repeated efforts to wash the central bodies from the surface of the membranes directly after impingement and prior to desiccation resulted in a 99% reduction in viability. Cyst and vegetative-cell suspensions similarly treated exhibited 100% viability. Further experimentation indicated that a great percentage of the central bodies impinged on the membrane were being inactivated by the impingement manipulation alone. The extreme fragility of these isolated cyst structures was shown by placing membranes on the surface of an agar plate immediately after impingement of the central bodies and permitting colonies to develop. The results indicated an inactivation of almost 80% of the central bodies placed on the membrane.

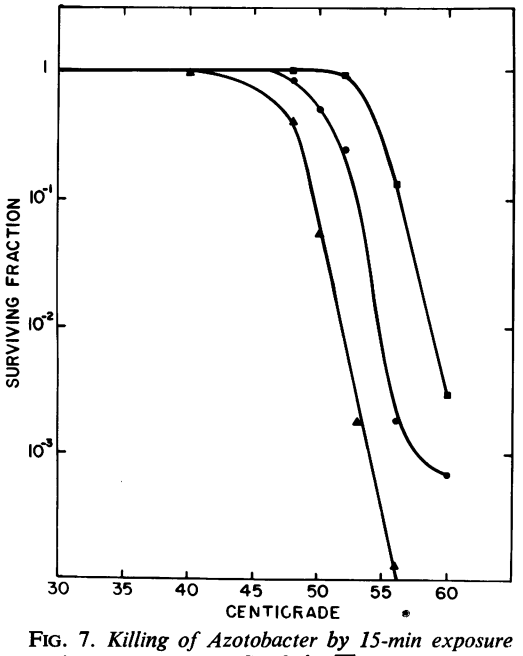

FIG. 7. *Killing of Azotobacter by 15-min exposure to various temperatures. Symbols:* ■ = *cysts;* ▲ = *vegetative cells;* ● = *central bodies.*

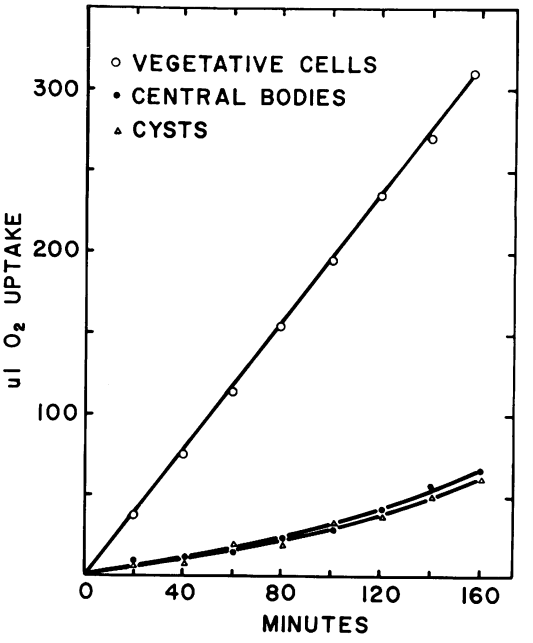

FIG. 8. *Respiratory activity of central bodies, cysts, and vegetative cells with 500 μmoles of glucose as the substrate. Oxygen uptake was expressed on the basis of 10^8 cells per milliliter. Endogenous activity of the cyst and central body preparations was nil.*

Respiratory capabilities of central bodies. Efforts to determine the oxidative capabilities of central bodies indicated they respire at about the same rate as intact cysts. Figure 8 illustrates the results of a typical experiment comparing the oxygen uptake of cysts, central bodies, and vegetative cells. Because of the volatile nature of butanol, glucose was used as a substrate. Previous experiments indicated butanol-grown cells respire without a lag when glucose is used as a substrate. It is observed that the amount of oxygen used by the cyst and central body suspensions is nearly equal and is only a small fraction of that used by a vegetative cell population of equivalent number.

DISCUSSION

The *Azotobacter* cyst is a remarkable structure exhibiting a tenacious vitality but retaining the ability to initiate vegetative multiplication when placed in a suitable environment. That the central body is the vital center of the encysted cell is shown by the properties it demonstrates when

extracted from the cyst coat. The evidence presented in this communication strongly suggests that the central body is a resting form of the vegetative cell with many of the same characteristics.

Extensive rupture of encysted cells can be effected by treatment with either EDTA or citrate. However, the action of these two chelating agents on the encysted cells is not identical. This is shown by the low survival rate of EDTA-treated cysts as contrasted with the high degree of viability displayed by citrate-treated cysts. It is feasible that the activity of citrate upon the encysted cells is primarily restricted to action on the cyst coat, whereas EDTA may, in addition, become involved in ancillary reactions involving the cell wall or vital intracellular processes. Further evidence for the gentle action of citrate on cells of *Azotobacter* is provided by treating the cysts with this compound during the various stages of germination. The fact that the concentrations of citrate employed for cyst rupture failed to produce a pronounced lethal effect on cells in any stage of germination indicates that the chelating agent did not interfere with cellular metabolism in an irreversible manner.

The retention of central bodies in the prefilter did not appear to be based solely on size, since the central bodies which passed through the filter were of the same dimensions as those observed in unfiltered preparations. Cysts, however, may be retained in the filter because of their large size. India-ink mounts of intact cysts reveal that there is a layer of polysaccharide slime exterior to the defined coat structure, indicating the entire cyst is actually much larger than might be expected from simple microscopic examination.

That the central body contains the vital parts of the cell is also illustrated by manometric experiments. Since cysts and central bodies take up oxygen at approximately the same rate when supplied with an exogenous carbon source, it can be assumed that the cyst coat serves merely as a protective covering and does not enter into normal cellular metabolism. These experiments also emphasize the fact that citrate has no permanent detrimental effect on cellular metabolism.

The relatively high sensitivity to ultraviolet light and sonic treatment of central bodies extracted from fully resistant cysts suggests that the coat is primarily responsible for the resistant properties of the cysts. Moreover, Socolofsky and Wyss (5) observed that the resistance of *Azotobacter* cells to ultraviolet radiation was acquired gradually during cyst formation, a process involving synthesis of the cyst coat, and was lost upon germination of the encysted cell.

Layne and Johnson (2, 3) described resistant forms of *Azotobacter* which appear to lack the complex coat generally characteristic of encysted cells. These authors induced formation of these resistant cells by omission of one or more minerals from the growth medium. They proposed that the cyst coat was not essential for marked resistance, since their forms which lacked this structure were extremely resistant to desiccation, sonic treatment, and ultraviolet irradiation. No alternative mechanism of resistance was suggested by these workers.

Our studies involving isolation of central bodies from resistant cysts have given no indication of any pronounced resistant properties of the cyst when the coat is removed. Furthermore, the gentle action of the citrate on the cysts has not suggested that the central bodies derived from the cysts are altered during the extraction procedures. In an effort to resolve the disparity between the results of Layne and Johnson (2, 3) and our own research, we have attempted to produce desiccation-resistant cysts by the method of mineral omission. To date these attempts have not been successful.

The results of our investigation indicate that the central body of the *Azotobacter* cyst is a resting form of the vegetative cell with many of the same properties. Since it is able to germinate and multiply when removed from the cyst coat, it can be assumed that it is an intact cell and is not comparable to a protoplast. The characteristic cyst coat appears to be primarily responsible for the resistance exhibited by the encysted cells, since without this structure the cells are as vulnerable as vegetative cells.

ACKNOWLEDGMENTS

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