THE MICROSPORIDIAN SPORE INVASION TUBE

The Ultrastructure, Isolation, and Characterization
of the Protein Comprising the Tube

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ABSTRACT

The extrusion apparatus of the microsporidian parasitic protozoan *Nosema michaelis* discharges an invasion (or polar) tube with a velocity suitable for piercing cells and injecting infective sporoplasm. The tube is composed of a polar tube protein (PTP) which consists of a single, low molecular weight polypeptide slightly smaller than chymotrypsinogen-A. Assembled PTP tubes resist dissociation in sodium dodecyl sulfate and brief exposures in media at extreme ends of the pH range; however, the tubes are reduced by mercaptoethanol and dithiothreitol. When acidified, mercaptoethanol-reduced PTP self-assembles into plastic, two-dimensional monolayers. Dithiothreitol-reduced PTP will not reassemble when acidified. Evidence is presented which indicates that PTP is assembled as a tube within the spore; that the ejected tube has plasticity during sporoplasm passage; and, finally, that the subunits within the tube polymer are bound together, in part, by interprotein disulfide linkages.

Microsporidian protozoa are intracellular parasites commonly found in a wide variety of animals. They are best known as parasites of arthropods (19), fishes (13), and mammals (12). The unique feature of these parasites is the extrusion apparatus found within the infective spore stage. When the spore is properly stimulated, this apparatus extrudes a long, fine tube which pierces the host cell and serves as the vehicle by which the sporoplasm is transferred into host cytoplasm (18). Tube discharge is an explosive event. Lom and Vavra (9, 10) showed that, in an appropriate hatching medium, discharge could occur within 0.1 s; further, these workers noted that the velocity of discharge was governed by the osmolarity of the incubation medium surrounding the spores (10).

Tube discharge from spores has a cinematographic resemblance to the generation of actin-rich acrosomal processes by certain stimulated sperm (14, 15); however, as this report will demonstrate, the microsporidian spore discharges a process composed of a single polypeptide assembled as a polymer with a two-dimensional lattice in the form of a tube.

MATERIALS AND METHODS

Collection of Spores

Muscle, heavily infected with *Nosema michaelis*, was removed from blue crabs *Callinectes sapidus*, added to 0.1 N KOH, and roughly homogenized with a Waring blendor for 30–60 s. The suspension was spun at 8,000 g for 20 min. The top material (mostly muscle homogenate) was decanted, and the crude spore pellet was

resuspended in water and centrifuged at 1,000 rpm for 10 min. The 1,000-rpm centrifugation and wash cycle was repeated until a pure white spore sediment was obtained. The purity of the pellet was checked by light microscopy.

Induction of Spore Discharge

N. michaelis spores were primed for extrusion by 90–120 min of incubation in freshly made Michaelis Veronal-acetate. This buffer was prepared by adding 9.7 g of sodium acetate and 14.7 g of sodium barbiturate in carbon dioxide-free distilled water to a final volume of 500 ml. After centrifugation, the spores discharged when placed on medium 199 with glutamine and Hanks' salts (Grand Island Biological Co., Grand Island, N. Y.).

Isolation of Polar Tube Protein (PTP) from Hatched and Undischarged Spores

Discharged spores were washed in 1% sodium dodecyl sulfate (SDS) and centrifuged at 1,000 g; the supernate was decanted and the pellet was resuspended in 2–3% SDS at neutral pH. In the presence of SDS, tubes of PTP remained assembled and attached to the discharged spores. This cycle was repeated until all surface tubulin protein from the spore walls (2) and the sporoplasm proteins were solubilized and washed free from discharged tubes. Finally, the purity of the pellet preparation of discharged tubes was examined by electron microscopy.

For the isolation of PTP from SDS-washed unhatched spores, the spores were washed in 8 N sulfuric acid at 95–100°C. The acid wash removed all the spore components except the wall and the assembled tube of PTP.

SDS-washed discharged spores, or unhatched spores treated with sulfuric acid, were treated with 50% 2-mercaptoethanol (2-ME) or with 1.0% dithiothreitol (DTT) for 16 h at 20°C. These reagents reduced PTP; however, ultrastructural examinations of the preparations indicated that the spore wall was unaffected. Reduced PTP, separated from spores by passing through a 0.45-μm filter (Nalge Co., Nalgene Labware, Div. of Sybron Corp., Rochester, N. Y.), was dialyzed for 24 h against Tris buffer and concentrated by lyophilization.

Disk Electrophoresis

PTP samples were separated in 5% polyacrylamide gels containing 0.1% SDS following the procedures of Weber and Osborn (17) and Dwyer and Weidner (2). Proteins were separated in 5 and 10 mM Tris-glycine and Tris-HCl buffer, pH 8.3 (3). Gels were 6 mm in diameter and 100 mm in length. Bromphenol blue, 0.001% (wt/vol) in buffer, served as the migrating front marker. Gels were fixed and stained for 2 h in 0.25% (wt/vol) Coomassie brilliant blue R_{250}, in 50% (vol/vol) methanol, and 7.5% (vol/vol) glacial acetic acid according to Weber and Osborn (17).

For estimating the molecular weight the isolated PTP

was reduced in 50% 2-ME, and the free sulfhydryl groups were alkylated for 1 h at 20°C with 0.5% sodium iodoacetate (11). This material was dialyzed for 24 h against the buffers used for electrophoresis separations. Reduced and alkylated protein was separated in 5% polyacrylamide gel. Aliquots of known proteins were used as standards (all obtained from Sigma Chemical Company, St. Louis, Mo.): chymotrypsinogen-A (bovine pancreas, crystalline), ovalbumin (crystalline), and bovine serum albumin (crystalline). The mobilities of the proteins were determined by the formula given by Weber and Osborn (17). In each instance, the mobilities were plotted against the log of the known molecular weight and expressed on a semi-log scale.

Immunodiffusion

Antibodies to hatched *N. michaelis* spores were prepared in white female rabbits. Weekly subcutaneous injections of hatched spores were administered with Freund's complete adjuvant. After 2 mo, the rabbits were bled by cardiac puncture. The sera were partially purified by 50% ammonium sulfate saturation, and the globulin was dialyzed against 0.15 M NaCl, 0.02 M Tris HCl pH 7.8, and stored at −20°C. These sera were run against isolated and reduced PTP on double immunodiffusion plates (Hyland Div. Travenol Laboratories, Inc., Cosa Mesa, Calif.).

Light Microscopy

Spore preparations were studied with Nomarski interference and phase optical systems. Light microscopy was useful in following the self-assembly of reduced and purified PTP.

Electron Microscopy Procedures

Negatively stained material was prepared by applying PTP preparations on Formvar-coated grids. Grids were passed through 2% (wt/vol) phosphotungstic acid (with a trace of bovine serum albumin) at pH 6.5, dried, and examined.

For surface views of discharged spores, platinum-carbon replicas were prepared by the procedure described by Koo et al. (7). Spore preparations were placed on Formvar-coated grids. The grids were shadowed at a 45° angle with platinum-carbon, and at a 90° angle with carbon. The Formvar was dissolved from the grid with acetone, and the replica was floated onto 50% Clorox in distilled water overnight, air-dried, and examined with an electron microscope.

Isolated spores and discharged tube preparations were fixed in 2% glutaraldehyde in 0.15 M sodium cacodylate buffer at pH 7.2. Spores and discharged tube pellets were postfixed in 2% (wt/vol) osmium tetroxide in the same buffer and processed by standard procedures (2).

Amino Acid Analysis

Samples were hydrolyzed in 6 N HCl for 24 h at 110°C. Analysis was performed with a Beckman model

119 Analyzer (Beckman Instruments, Inc., Fullerton, Calif.), using single column methodology. Half-cystine was determined as cysteic acid following performic acid oxidation.

RESULTS

Microsporidian spore morphology is presented as background to observations on the nature of the protein in spore discharge tubes (Figs. 1 and 2). The spore stage of *N. michaelis* and *Nosama lophii* is composed of sporoplasm, an extrusion apparatus and spore wall. The cytoplasmic component of the sporoplasm is dispersed around, but exterior

to, the extrusion apparatus (Fig. 3). The most obvious component of the apparatus is the polar filament; this structure is a long, membrane-bound coil consisting of a densely packed core (Fig. 3). The perimeter of this core resists washing in 8 N sulfuric acid and appears to be equivalent to the discharged tubes (Figs. 6 and 7). Another component of the extrusion apparatus is an assemblage of membranes termed the polaroplast. In *N. lophii*, the polaroplast consists of pleated membrane wrapped around the ascending portion of the polar filament (Fig. 3).

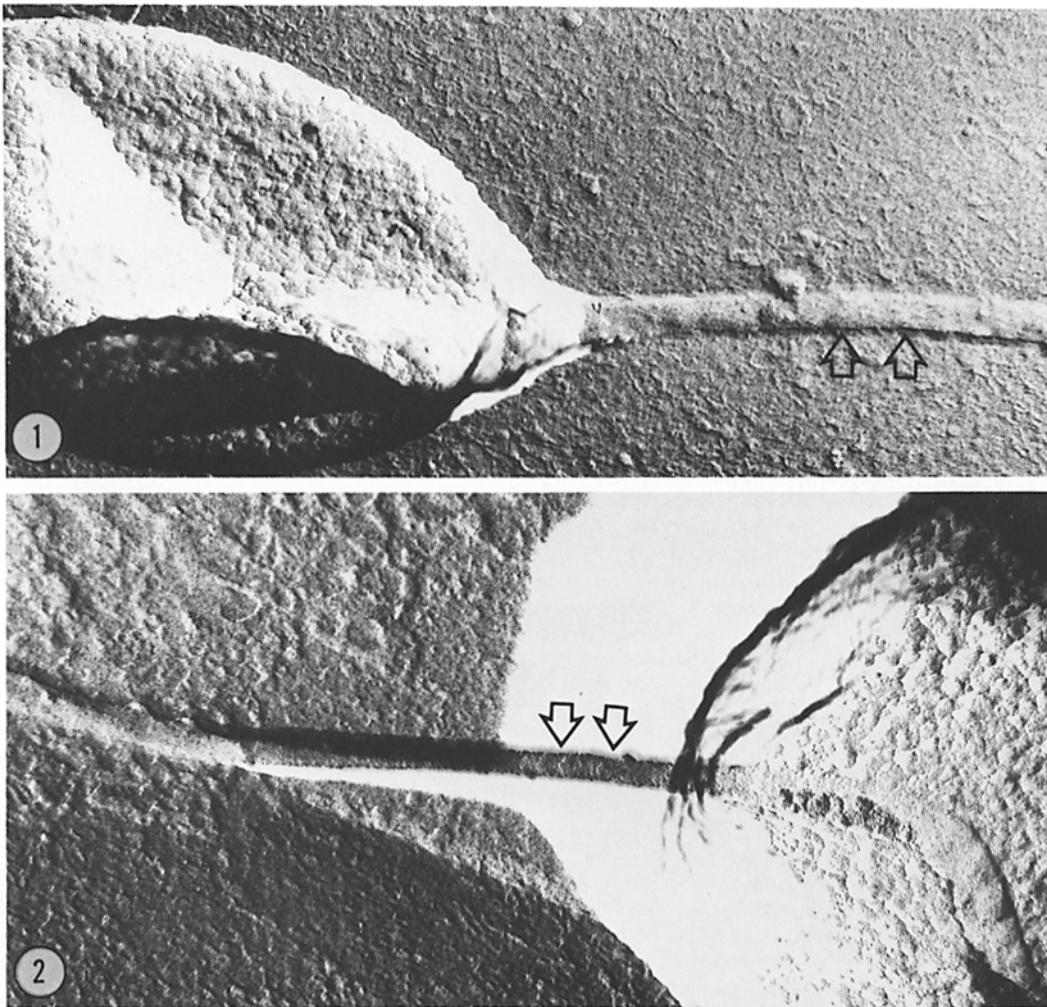*N. michaelis* spores average 1–1.2 μm in length.

FIGURE 1   Platinum-carbon replica of *N. michaelis* spore with discharged tube (arrows). Tubes of *N. michaelis* spores have a uniform 100-nm thickness and a 50-μm length. $\times$ 50,000.

FIGURE 2   Platinum-carbon replica of discharged tube extending to and over the surface of a spore (arrows). Discharged tubes have some flexibility but tend to retain the 100-nm thickness. $\times$ 40,000.

Spores, primed in Veronal-acetate buffer, discharge a tube 50 μm in length and 100 nm in width in a fraction of a second; after 5–30 s, the sporoplasm accumulates in a vesicle at the distal end of the discharged tube. After discharge, only the spore wall, plasma membrane, and some membrane profiles remain in the spore ghost (Fig. 4). Concentric membrane profiles were observed in the spore ghosts in positions where the membrane-bound polar filament was situated before discharge (Fig. 4).

The thickness of the polar tube was 100 nm before and after discharge (Figs. 5, 7, and 9). Tube thickness remained approximately 100 nm although spore discharge was tested in media with different viscosities ranging from distilled water to 1.0% gelatin. Tubes of *N. lophii* remained at 100 nm thickness whether extrusion was through the side of a spore or through the aperture. However, discharging tubes showed variations in thickness up to 350–400 nm at loci where sporoplasms moved through tubes.

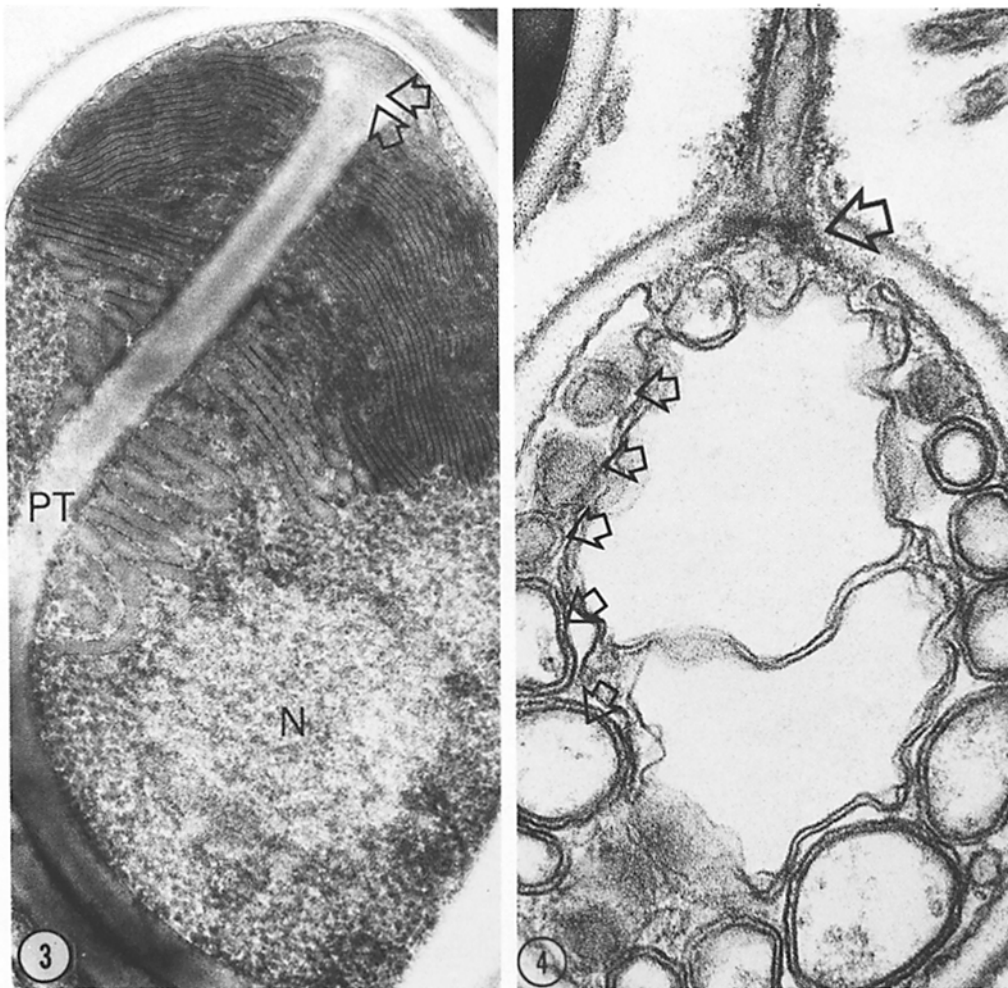The microsporidian wall was resistant to SDS, 2-ME, and DTT. Undischarged and discharged

FIGURE 3   *Nosema lophii* spore. Tangential section through ascending loop of polar tube (PT). Nucleus (N), diffuse cytoplasm, and polar aperture (arrows). × 60,000.

FIGURE 4   *N. michaelis* spore ghost after discharge. This section shows membrane profiles (arrows) in positions within spore ghost where coil of membrane-bound polar filament was located in undischarged spore. Note small exit point at polar aperture (large arrow). × 80,000.

FIGURE 5   Negatively stained discharged tubes of *N. michaelis*. Polar tube protein (PTP) is organized as a cylindrical monolayer. The material in the lumen is SDS-soluble and presumed to be sporoplasm passing through the discharge tube. × 150,000.

tubes of PTP resisted dissociation in 1–3% SDS, in brief 5-min washes in 8 N sulfuric acid at 100°C, and across the pH range. Tubes of PTP of unhatched and discharged spores were dissociated with 2-ME and DTT. Because of the resistance of tubes to SDS and to washings with 8 N sulfuric acid, the PTP material was easily purified from other soluble components within or outside of the spores (Figs. 6–9).

Assembled PTP of unhatched spores, washed with 8 N sulfuric acid at 100°C for 5 min, had an appearance similar to that of the SDS-washed discharged tubes (Figs. 7 and 9). The assembled PTP, whether in the tubes inside unhatched spores or in discharged tubes, was composed of subunits which were below the resolution limits of our detection system for accurate assessment of size and shape. However, assembled PTP had a two-
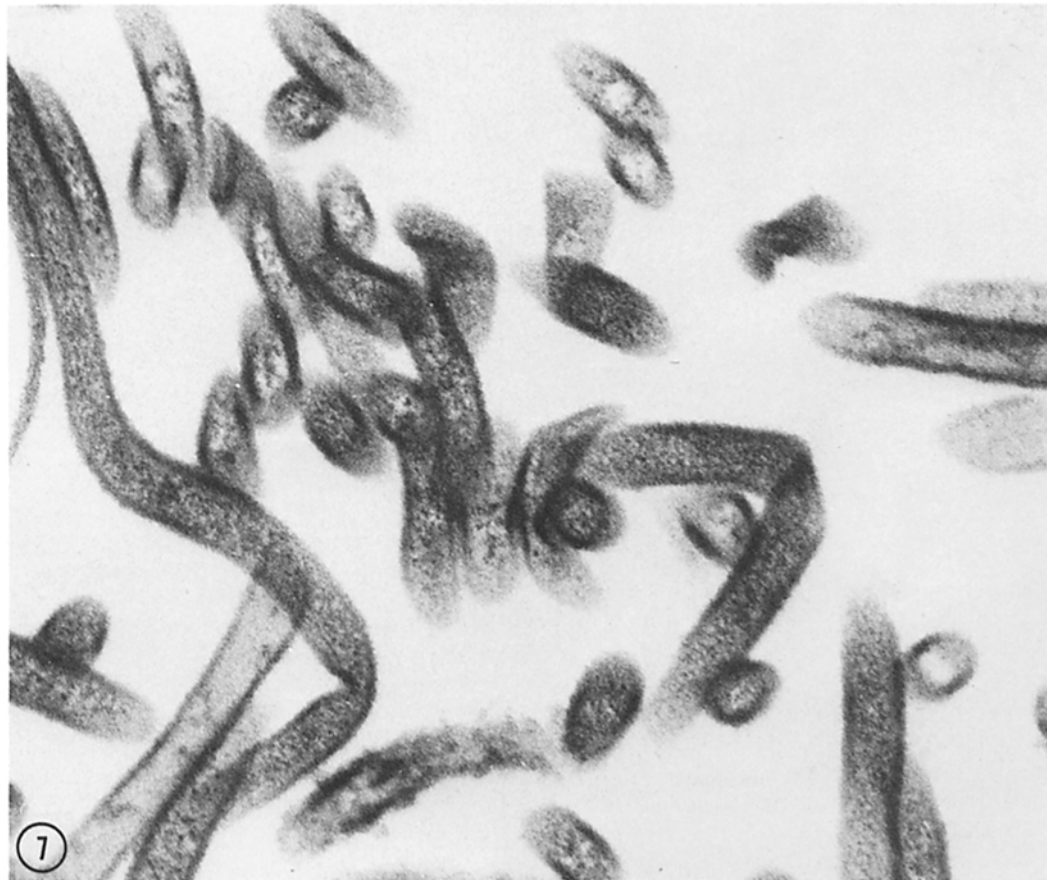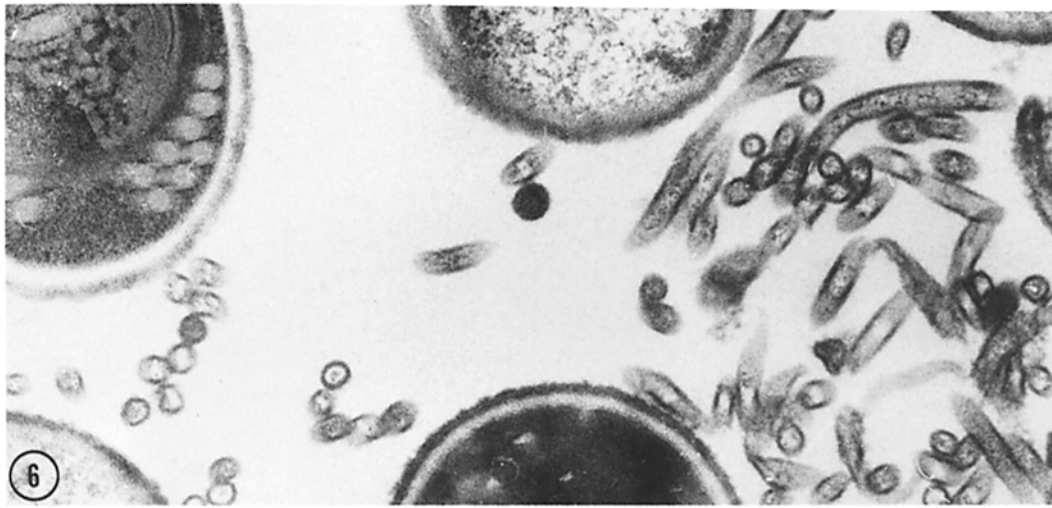
FIGURE 6 *N. michaelis* pellet of SDS-washed discharged spores and tubes. × 30,000.

FIGURE 7 Higher magnification of *N. michaelis* pellet of SDS-washed discharged tubes. Note bends or folds in tubes and the absence of material in the lumens of the tubes (compare to Fig. 5). × 100,000.
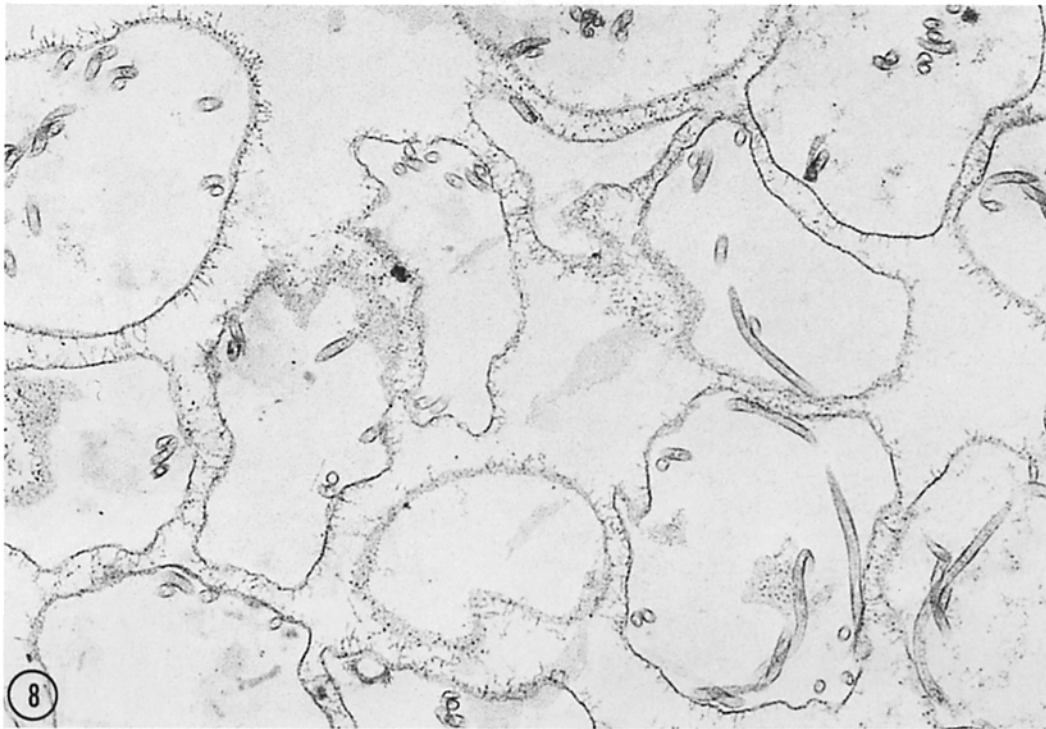
FIGURE 8 *N. michaelis* spores treated with 8 N sulfuric acid at 100°C for 5 min. The spore wall and undischarged polar tube are the only structures that resist sulfuric acid treatment. × 20,000.

FIGURE 9 *N. michaelis* spore treated with 8 N sulfuric acid at 100°C for 5 min. Note tubular nature of the discharge tube. This condition is not apparent in standard preparations of spores (see Fig. 3). × 80,000.

dimensional construction, particularly noticeable in SDS-washed discharged tubes and in the tubes within unhatched spores washed with sulfuric acid. Centrifuged pellets of SDS-washed tubes displayed a uniform thickness but were characteristically twisted or folded (Fig. 7).

The Action of 2-ME and DTT on PTP

When pelleted SDS-washed tubes of PTP were solubilized with 2-ME, unalkylated, and dialyzed against alkaline buffer, PTP remained dissociated; however, PTP reaggregated when the solution was

acidified (Figs. 13–15). If 2-ME-reduced PTP was alkylated with iodoacetic acid, the PTP remained dissociated. On the other hand, when SDS-washed tubes were solubilized with DTT, the PTP did not reassemble after removal of the reducing agent and after acidification of the solution.

Mercaptoethanol-reduced and alkylated PTP, or DTT-reduced PTP, formed a single, distinct band with SDS gel electrophoresis (Fig. 10). The PTP band was similar, but had a slightly faster mobility than the standard, chymotrypsinogen-A. If 2-ME-reduced but unalkylated PTP was acidified, no band was obtained with SDS gels; however, if DTT-reduced PTP was adjusted to an acid pH, an electrophoretic band formed with a mobility equivalent to that of the band obtained with 2-ME-reduced and alkylated PTP. The PTP had a mean relative mobility (R_M) of 0.75 ± 0.03. On the basis of eight individual determinations, the mean molecular weight of PTP was estimated at 23,000 (Fig. 11).

Double immunodiffusion gels showed a single precipitin line between 2-ME-solubilized PTP and rabbit serum against discharged *N. michaelis* spores (Fig. 12).

Isolated PTP, checked electrophoretically for purity, was subjected to amino acid analysis (Table I). The estimation of the number of glycine residues may be slightly high since the PTP monomers were originally stored in a Tris buffer containing some glycine; however, the sample was exhaustively dialyzed to remove this residue before proceeding to hydrolysis of PTP to amino acid residues.

FIGURE 11 Molecular weight plot of reduced and alkylated PTP separated in 0.5% gels containing 0.1% SDS. Marker proteins were: chymotrypsinogen-A (average mol wt 25,000; ovalbumin, 45,000, bovine serum albumin [bsa], 67,000). Ovalbumin (ovalb.) dimer, mol wt 90,000, and bsa dimer, 134,000, were also plotted. PTP is indicated as a monomer.
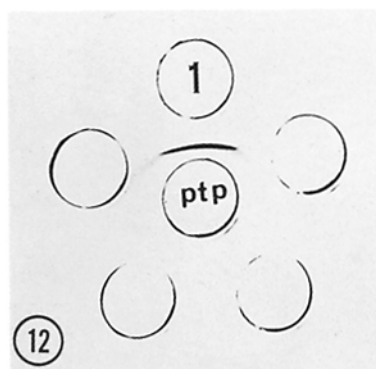
FIGURE 12 Double immunodiffusion of 2-ME solubilized and purified PTP against partially purified rabbit anti-*N. michaelis* (hatched spores) globulin (1). The immunodiffusion was carried out for 48 h at 22°C.

Behavior of Self-Assembled PTP

When 2-ME-reduced PTP was acidified, the monomers reassembled into sheets or closed shells. Nomarski interference microscopy showed that the assembled material had a viscous consistency (Figs. 13–15). We noted with phase microscopy that the particles incorporated into these PTP shells moved about freely inside. Negative staining of self-assembled PTP showed a lattice-like periodicity similar to that of thin-sectioned discharged tubes (Figs. 16, 16*a*, 16*b*).
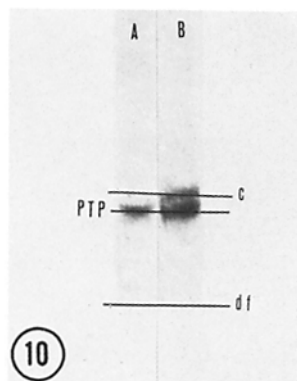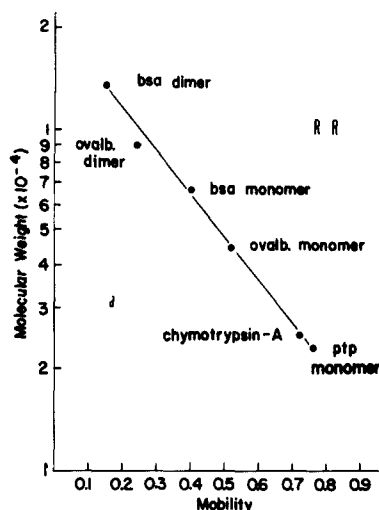
FIGURE 10 SDS gel electrophoresis using Tris-glycine as the running buffer. Left gel (A) is 2-ME-solubilized and alkylated PTP. The right gel (B) is PTP combined with the standard, chymotrypsinogen-A (C). Dye front is indicated by base line (df).

DISCUSSION
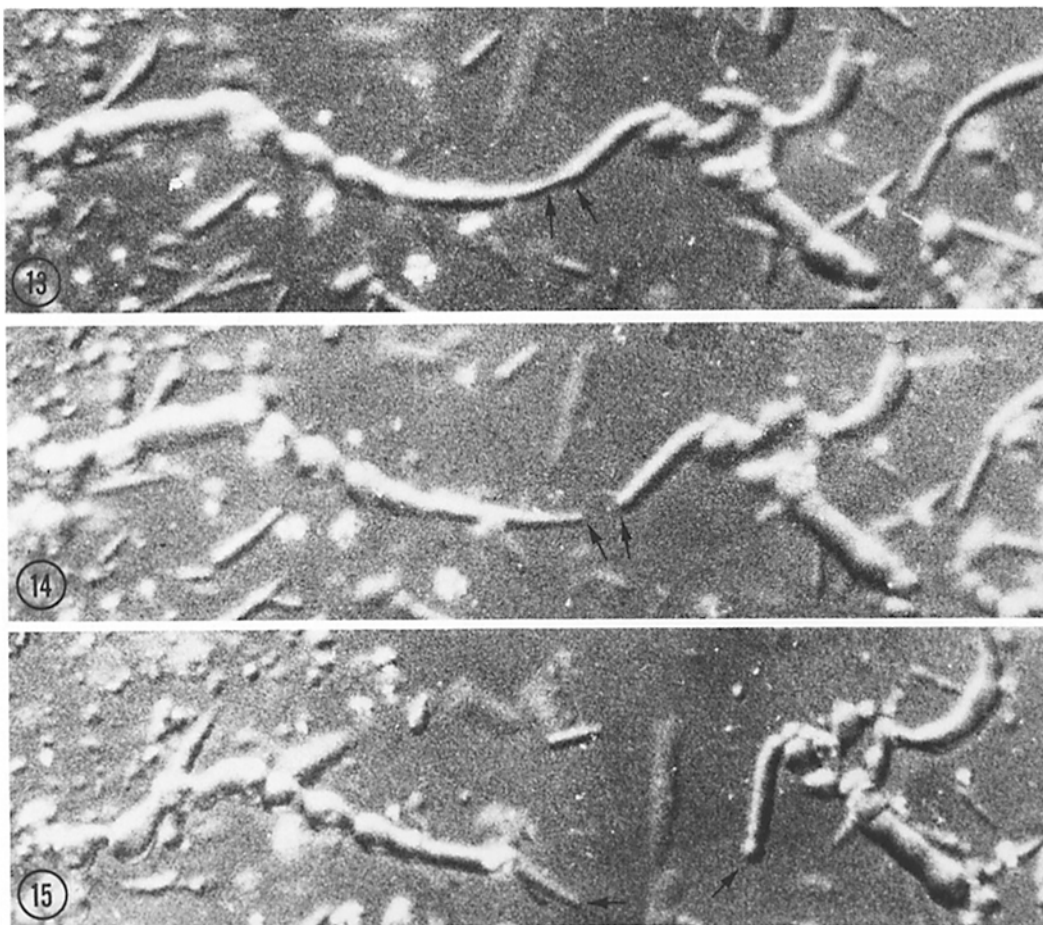
Assembled PTP is in the form of a tube in un-hatched spores washed free of a core component with 8 N sulfuric acid. Since the PTP tube is

TABLE I

Amino Acid Composition of PTP

Number of residues ± 1/protein molecule			
Alanine	16	Aspartic acid	19
Valine	15	Glutamic acid	25
Isoleucine	10	Lysine	12
Leucine	13	Arginine	7
Proline	7	Serine	15
Phenylalanine	6	Threonine	4
Methionine	2	Half-cystine	5
Glycine	30	Tyrosine	8
Tryptopan	—	Histidine	4

structurally indistinguishable from the core material inside the tube, it is believed that the core may be an unassembled form of PTP. Assembled PTP of unhatched and discharged spores exhibits a strong protein-protein interaction which resists dissociation in SDS and in media at the extreme ends of the pH range.

The following evidence indicates that the binding between PTP subunits is due to interprotein disulfide linkages: First, when assembled PTP is dissociated with 2-ME and is left unalkylated, the subunits reassemble. This behavior is characteristically observed when S-S bridges are split with 2-ME. When 2-ME is removed, the disulfide bridges are free to reform (4). Second, when assembled PTP is dissociated with DTT, the PTP subunits do not reassemble after removal of the

FIGURES 13, 14, and 15 Nomarski interference optics of self-assembled PTP. Figs. 13, 14, and 15 were taken at 40–60-s intervals. Stock of 2-ME-reduced and unalkylated PTP was previously acidified to induce self-assembly. The fluidity of self-assembled PTP is indicated by arrows. × 2,000.
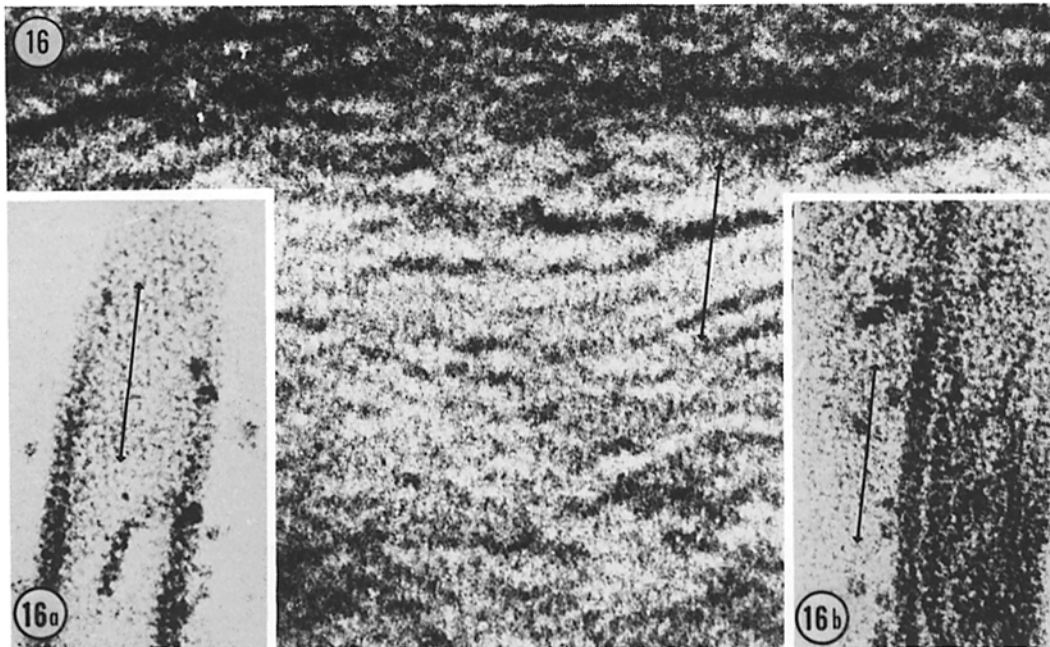
FIGURE 16 Negatively stained preparation of self-assembled PTP. PTP formed monolayers on Formvar-coated grid. × 200,000 (a, b). Longitudinal thin sections through discharged tubes. Note that the periodicity is similar to that of the negatively stained preparation in Fig. 16. × 200,000.

reducing agent. DTT has a low enough redox potential to maintain thiol groups in a reduced state (1), and thus, it successfully prevents reassembly of PTP subunits. Finally, five half-cystine residues are found in each molecule of PTP. Half-cystine residues are likely involved in the interprotein S-S bridging since 2-ME and DTT readily dissociate the PTP polymer into identical subunits.

A number of observations indicate that one polypeptide is present in the discharged tube of PTP. First, with SDS gel electrophoresis, one band is produced with DTT-reduced PTP and with 2-ME-reduced and alkylated PTP. Second, one reaction band formed in double immunodiffusion gels with solubilized PTP tested with antisera prepared against hatched N. michaelis spores. Finally, 2-ME-reduced PTP assembles into sheets or shells. According to Kellenberger (6), small, identical polypeptide monomers characteristically assemble into polymers constructed in the form of a two-dimensional lattice. I observed the two-dimensional nature of PTP in the tubes washed with SDS, and in the PTP in unhatched spores washed with sulfuric acid. Finally, with phase microscopy, purified and dissociated PTP self-assembles into sheets or closed shells. When particles became entrapped inside these shells, the particles dis-

played a free motion; such a motion would be expected if particle material was confined rather than suspended in a substance.

Self-Assembly of PTP

When 2-ME-reduced PTP is acidified, it self-assembles. By negative staining, the self-assembled sheets resemble the PTP in discharged tubes. A considerable difference exists in the degree of plasticity between self-assembled PTP and the protein in discharge tubes. The PTP in tubes is only slightly plastic during sporoplasm passage; the limited flexibility of these tubes is well illustrated in the pellets of tube material folded and twisted by centrifugation. On the other hand, self-assembled PTP has a behavior of a two-dimensional viscid substance with little tendency to conform to any particular shape. I suspect that a stabilizing component is missing, or perhaps the self-assembled subunits have less order than the units in spore discharge tubes.

How Does PTP Come Out of the Spore at Discharge Time?

An ultrastructural view of sulfuric acid-washed spores indicates that PTP exists as a tube before

discharge. During the past two decades, it was believed that the membrane surrounding the intraspore PTP was an important constituent of the discharged tube (10, 16). However, my observations indicate that this membrane remains behind in the spore ghost. Another concept which I now question is that the discharge tube everts while moving out of the spore (10, 18). The following observations make tube eversion seem unlikely: First, one would expect two layers of PTP at the distal end of an everting tube; what one sees is a single layer of PTP before, during, and after tube discharge. Second, it would seem unlikely that the discharging tube could have a piercing capacity (18) if PTP was everted at the distal end. Third, discharging tubes have a consistent shape with little variation, regardless of the chemical or physical forces applied to the discharging spore. If discharge was accomplished by eversion, the shape of the tube would likely be altered by changes in the osmolarity or viscosity of the external medium. Fourth, when spores are fixed briefly in glutaraldehyde, the PTP material appears affected. Tube discharge after this treatment shows that the tube retains the coiled arrangement it had in the spore. It is unlikely that crosslinked PTP could retain a coiled profile after PTP subunits were everted during tube discharge. Finally, a substantial energy requirement would be expected for executing the rapid release and change in the order of the PTP polymer through eversion, since the protein-protein subunit interaction within the tube is strong.

It is suspected that assembled PTP is ejected as a tube after the buildup in osmotic pressure within the spore. Lom and Vavra (10) noted that the velocity of tube discharge was proportional to the osmotic pressure buildup within the spore. Apparently, all of the tube must be ejected before sporoplasm passage; sporoplasm movement through the tube was not observed when the PTP was only partially ejected.

Sporoplasm Passage Out of the Spore

It has been substantiated by various workers (5, 8, 18) that microsporidian spores inoculate infective sporoplasm through an invasion tube and into cells. The sporoplasm is ejected from the spore when the tube discharges rapidly and when the external medium is not hypo-osmotic. *N. michaelis*, *N. lophii*, and *Glugea hertwigi* will discharge a tube, but the sporoplasm will not pass through it if the medium is hypo-osmotic. For sporoplasm ejection from these spores, the external medium requires a certain consistency such as is found in cell culture medium, cell cytoplasm, or plasma.

The apparent uncompartmentalized condition of the cytoplasmic component of the sporoplasm may be the reason why movement of sporoplasm from the spore to the end of the tube is scarcely detectable; the tube shows only a gradual two- to threefold increase in thickness at points where the sporoplasm passes.

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