CALI, Ann, 1943-
A FINE-STRUCTURAL STUDY OF NOSEMA APIS ZANDER
(MICROSPORIDA : CNIDOSPORA : PROTOZOA).

The Ohio State University, Ph.D., 1970
Entomology

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1971
A FINE-STRUCTURAL STUDY OF NOSEMA APIS ZANDER
(MICROSPORIDA : CNIDOSPORA : PROTOZOA)

DISSERTATION

Presented in Partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy in the
Graduate School of The Ohio State University

By
Ann Cali, A.A., B.S., M.S.

The Ohio State University
1970

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FIELDS OF STUDY

Major Field: Invertebrate Pathology
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INTRODUCTION

The term microsporidia is used for a group of parasitic protozoa reported as obligate intracellular parasites of every major phylum of organisms in the animal kingdom, from other protozoa to vertebrates. The only comprehensive review of hosts of the microsporidia was compiled by Kudo in 1924.

The first microsporidian was described by Nageli in 1856. This microorganism, Nosema bombycis, was shown to be the causative agent of pebrine, a disease of silkworms, by Pasteur (1870).

In 1882 Nosema bombycis and other morphologically similar organisms were grouped together as the Microsporidia without a designation as to taxon (Balbiani, 1882) in the Class Sporozoa.

Nosema apis, a parasite of the honey bee (Apis mellifera) and consequently of economic importance, was described by Zander (1909). It has shared, with N. bombycis, the attention of researchers as the most frequently documented microsporidian.
Several thousand papers have been written on *N. apis* as a parasite of honey bees. However, only a few hundred could be classified as experimental research on the organism or effect on the host. Several investigators (Morganthaler, 1922; Kudo, 1924; White, 1919; and Zhandov, 1960) have published comprehensive reviews of the literature on *N. apis*.

Balbiani's student, Thelohan, placed the Microsporidia in the order Myxosporidia and called them the family Glugeidae (Thelohan, 1892). Gurley (1893) upgraded the group to order and named it order Cryptocysts and in 1899 Labbe went back to Balbiani's original name for these organisms in his classification system and created the order Microsporidia.

From 1899 until 1964, the microsporidia were referred to as an order in the Class Sporozoa. A revision of the phylum Protozoa was published in 1964 (Honigberg, et al., 1964). In the revision, the microsporidia were removed from the Class Sporozoa and placed in a new sub-phylum, the Cnidospora:

Sub-phylum Cnidospora

Class Microsporida

Order Microsporida

Sub-order Monocnidina

The organisms commonly referred to as the microsporidia are those in the sub-order Monocnidina.
Much new information has been acquired on the microsporidia (Monocnidina) within the past ten years as a result of technological advancements in microscopy, more specifically to the increased availability of the electron microscope. Table I provides a guide to the literature dealing with the fine structure of the microsporidia. The Table shows that most of the ultrastructural investigations concern the spore, revealing a need for further studies on the proliferative stages, frequently referred to as the vegetative forms.

The present study concerns the entire life cycle, proliferative as well as spore stages, of *N. apis*. The light microscopy is covered briefly, with emphasis placed on the general appearance of the parasite as it goes through the various phases of development from sporoplasm to spore within the midgut epithelial cells of host honey bees (*Apis melifera* L.).

The electron micrographic coverage deals with the detailed changes in structure during the development of the parasite within the host.
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<td>X</td>
<td><em>N. cuniculi</em>; Vertebrate hosts review paper</td>
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MATERIALS AND METHODS

Facilities

The honey bees used for all experiments conducted in this study were obtained from The Ohio State University Bee Laboratory.

One colony with a high incidence of Nosema apis was maintained in a glass enclosed (11' x 22' x 12') greenhouse room. This hive, along with an uninfected control colony in another similar room of the greenhouse, was used for the initial experiments to determine the life cycle of N. apis as observed with the aid of light microscopy. For specific methods and materials see Gray, Cali, and Briggs (1969).

In addition to information on the timing and the nature of the life cycle of N. apis the infected colony in the greenhouse was maintained as a source of N. apis inoculum.

Experimental Bees

Frames of sealed brood obtained from healthy colonies maintained at the bee yard were placed in a wire screen sealed container and incubated at 31°C for a 24 hour period. Three hundred-fifty bees that emerged during the 24 hour period were paint marked with Testor's quick drying enamel
paint (white for infected bee hive and green for control bee hive) and placed in two miniature observation hives (each containing one comb 6" x 8½"). The combs in the miniature hives each contained newly sealed brood and pollen cells. A sugar syrup feeding bottle and a water bottle were placed on each hive over a screen so the bees would be able to feed but not fly out of the hive. The hives were incubated at approximately 31°C for the duration of the experiment. Three days after the experimental bees (paint marked) were put into the miniature hives, a super containing a comb of unsealed brood was put on each hive, so the experimental bees would be able to perform normally as nurse bees.

When the experimental bees were about one week old the sugar solution bottle on the hive containing the white marked bees was replaced by a bottle of sugar water containing *N. apis* spore as inoculum (3 x 10⁷ spores/ml).

**Sampling Procedure**

Experimental bee samples, each of 5 animals, were taken at 9:00 A.M. and at 4:00 P.M. for processing for light and electron microscopy. This sampling was continued from the day of inoculation through the following 7 days.

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1 Testor’s paint may be obtained in any hobby store.
Processing of Experimental Bees

For Light Microscopy:

Each bee was dissected, the ventriculus removed, and fixed in modified aqueous Bouin's fluid for 24 hours, then transferred to 70% Ethanol for storage. The ventriculi were dehydrated and embedded in Ester wax in a vacuum oven, sectioned at 4 μ and stained with Heidenhain's haematoxylin according to the methods and schedule given in Appendix I.

For Electron Microscopy:

The ventriculus was dissected from each bee and immediately placed in ice cold buffered glutaraldehyde-fixative. The ventriculus was cut into several small pieces using two razor blades, and placed into vials containing ice cold glutaraldehyde. Four hours later the tissues were post fixed in osmic acid (MILLINOG, 1961) for 6-8 hours, dehydrated, and embedded in epoxy resin. Sections were cut on a Sorvall Porter-Blum MT-2 ultra microtome using a Dupont Diamond knife. Only gray to silver sections were used. The sections were picked up on 100 mesh carbon and parlodion coated grids or 300 mesh uncoated grids, stained with uranyl acetate and lead citrate, then viewed with the aid of an R.C.A. EMU3G electron microscope. Details of electron microscopy methods with the modifications used may be found in Appendix II.
Photography

Light micrographs were taken on 35 mm fine grain panatomic x film with an ASA rating of 32. All film was developed in Microdol x developer and printed on Kodak Kotabromide paper F1 to F5.

Kodak or Illford 2" x 10" glass plates were used for electron micrographs and developed on Kodak or Agfa paper depending on the contrast needed.

Magnifications specified in legends for electron micrographs are those of micrographic prints.
RESULTS

Light Microscopic Evaluation of the Morphogenesis of \textit{N. Apis}

Before the developmental phases of an intracellular obligate parasite can be adequately described, the normal morphology of the host cell and the location of the parasite in the host cell must be established. Figures 16 and 17 are micrographs of cells that are uninfected, cells containing sporoplasms, and heavily infected cells. The sporoplasm of the parasite is in every case located in the cytoplasm. von Steche (1965) asserted that \textit{N. apis} was located in the host cell's nucleus; however, even with a heavily infected cell (Figure 18) in which the entire cytoplasm was filled with spores, the nucleus was completely intact and uninfected.

The following is a description of developmental stages of \textit{N. apis} in the cytoplasm of honey bee ventricular epithelial cells as seen with the aid of the light microscope.

"A binucleate planont, which is believed to have emerged from the adjacent polar filament, is shown in Fig. 1. This particular planont was located in the lumen of the honey bee ventriculus and probably would not have resulted in a successful parasitization, since the filament did not penetrate an epithelial cell. Successful implantations are shown in Fig. 2 and 3. More than one planont may be found in an individual ventricular cell. Figure 4 shows the beginning of the first
division of the schizont; the two nuclei have migrated to opposite ends of the cell. Figure 5 shows the beginning of the formation of two binucleate schizonts. Eventually the schizonts form long chains (Fig. 6) from which the individual binucleate schizonts break off (Fig. 7). The parasite's nuclei at this stage are characterized by small, centrally located, heavily stained areas of chromatin, surrounded by nucleoplasm. Figure 8 shows the beginning of the formation of the second schizogonic chain which is characterized by heavily stained chromatin-rich nuclei. The nuclei may divide synchronously and yield a four-nucleate stage or they may divide asynchronously forming first a three-nucleate stage (Fig. 8), and then a four- or six-nucleate stage (Fig. 9). The four-nucleate stage appears to be more common (Figs. 10 and 11). It is while the four-nucleate stage is forming that the increase in chromatin-staining material becomes noticeable and the size of the parasite increases. For this reason, the quadranucleate vegetative stages are more frequently observed in smears and sections of gut cells, than the less conspicuous earlier stages. The quadranucleate forms divide, yielding two binucleate cells or diplokaryons (Figs. 11 and 12). The two nuclei of the diplokaryons remain adjacent during subsequent sporogenesis (Figs. 13 and 14). Figure 15 shows the final stage of parasite development, the mature spore.

Sometimes heavily infected ventricular cells are seen with the binucleate forms of both the first and second schizogonic stages, and no intermediate stages. This suggests that it may be possible for the binucleate forms of the first schizogony to develop directly into diplokarya and sporoblasts by omitting the quadranucleate stage."

(Gray, Cali, and Briggs, 1969)

**Electron Micrographic Evaluation of the Morphogenesis of N. Apis**

The sporoplasm has a thin, lightly staining limiting membrane. The cytoplasm is rather granular in appearance
containing two distinctive organelles; short segments of rough endoplasmic reticulum and nuclei. The nucleus as seen in Fig. 20 contains a rather distinct nucleolus.

The sporoplasm (Figs. 19 and 20) appears to be mononucleate, not binucleate as seen in the light micrographs, as only one of the nuclei was in the plane of the tissue section. In addition, a section of tissue cut for viewing on the electron microscope is approximately 0.025μ thick, while the sections illustrated in the light micrographic part of this study were sectioned at about 2 to 4μ in thickness. The probability of getting a section through both nuclei, as in the light micrographs, is consequently much smaller.

After having been emplanted in the cytoplasm of an epithelial cell, the sporoplasm begins to elongate and increase in nuclear number. Figure 21 is a low magnification (7,000x) micrograph of a cross-section through a ventricular epithelial cell. This micrograph illustrates the location and appearance of the elongating schizont developing within the cytoplasm of the host cell. The schizont displaces the organelles of the host cell. The nuclear membrane in Figure 20 is recurved around the parasite. Figure 22 (18,400x) is a higher magnification of the parasite (Fig. 21) which is at this point a long convoluted structure appearing as 3 to 4 separate parasite cells due to the cutting angle. A comparison of Figure 22
and Figure 23, which are micrographs of different sections through the same host cell, gives a three-dimensional view of the parasite.

Figure 24 is a micrograph through an epithelial cell, containing a long-chain schizont. The parasite is sectioned along the length of the chain. The nuclear arrangement in this proliferative "chain" as seen in Figures 25, 26, and 27 (higher magnification micrographs of parts of Figure 24) illustrate the paired nuclei in a stage which is judged to be prior to sporogony because the chain has not yet divided to form individual cells.

These nuclei divide yielding more pairs of nuclei (Fig. 25) until the chain occupies almost all of the cytoplasmic volume of the host cell. This type of long-chain structure is more clearly illustrated in Fig. 6 of the light micrographs. Nuclear division occurs by the formation of a spindle inside the nuclear membrane (Fig. 28), the entire division process taking place without the nuclear membrane breaking down.

While proliferating, the cell membrane of this elongated structure forms indentations (Fig. 6 - light micrographs and Fig. 25 - EM) where cell division eventually takes place producing individual binucleate cells of the parasite. By this time several noticable changes are taking place. There seems to be much more organelle structure in the cytoplasm than in the earlier stages (Figures 28, 29
and 30). There is an increase in the amount of rough endoplasmic reticulum in these cells, some smooth surfaced vesicles are present, and the nuclei are still joined. In the new individuals thus formed, the cell limiting membrane begins to thicken. In Figures 30 and 31 the membrane has become much thicker in some regions than in others. Figure 32 is a higher magnification micrograph of a portion of the membrane, eventually the entire membrane becomes thickened (Figs. 33 and 34).

Nuclear division continues occurring after the cell membrane has thickened, forming a second elongated structure (Figs. 35 and 36). The nuclei in these cells are in the process of division and the cell limiting membrane has already thickened.

When the final cell division has occurred, yielding binucleate heavy membraned forms, a new structure appears in the cytoplasm of each cell. It is located at one end of the elongated binucleate cell and is a round vesicular structure, seen in Figures 33 and 37.

A thin membraned irregularly shaped sac-like vesicle and a spherical vesicle with an electron dense periphery are attached to the vesicular complex (Figs. 33, 37, and 38).

Following the appearance of these structures is the appearance of polar filament (Fig. 39). In longitudinal sections of the parasite in this stage of development, the
... coils of the filament are seen formed around the vesicular structure (Figs. 40, 41, 42, and 43). The filament increases in number of coils as development proceeds. The parasite cell at this stage of development is illustrated in Figures 44, 45, 46, and 47. The base of the polar filament is located at the opposite end of the cell from the vesicular body and the coils of the polar filament. Figure 48 illustrates this vesicular structure as distinct from rough endoplasmic reticulum or the nuclei of the cell because all three are present in the micrograph. Microtubules are sometimes seen in association with this vesicular mass (Figs. 49 and 50). Two nuclei are present and joined to each other and rough endoplasmic reticulum is abundant throughout the cytoplasmic area. The thickened membrane completely surrounds the cell by this stage (Figs. 46, 47, 51, and 52). The figures mentioned above all illustrate the presence of the polar filament before the heavy layers of the spore coat are formed. Figures 41, 45, 46, and 47 are sections through the length of the cell, and demonstrate the general location and appearance of the organelles.

The spore of *N. apis* has been described by Schotylseck and Danneel (1962), by Codreanu, et al. (1965), and by Popa, et al. (1967). Schotylseck and Danneel's paper was primarily concerned with the structure and the length of the polar filament.

"These transverse sections are adequate to 44 windings of the polar filament, being up to about
250u in length. The polar filament is filled with fibrils, the arrangement of which as a whole resembles that in cilia and flagella."

Popa, Codreanu, and their coworkers have summarized their findings in English in Popa, et al. (1967):

"Under the electron microscope these sections show the outer membrane of the spores with three layers, the micropyle, the nucleus, the polaroplast, and other elements. It is equally to be observed in certain stages of evolution that the membrane is invaginated on the level of the micropyle and inside the adult spores the polar filament are closer together towards the equator of the spore, the number varies probably because of the different age of the spores, the maximum number being 35. On its transversal section, the polar filament has a circular outline, it seems to be constituted by two concentric layers surrounded on the outside by a thicker membrane. The exterior layer is provided with bundles of very small longitudinal fibres having in their turn an oval section. The images of the polar filament after expulsion present a uniform calibre."

The observations made in the present study are in agreement with the above mentioned reports on the nature of the spore coat. It is characterized by a thin outer region, followed by a broader electron transparent area and a thin electron dense area which surrounds the interior structure of the spore (Figs. 53, 54, 55, and 58). The number of polar filament coils vary from spore to spore (34 in Fig. 56; 14 in Fig. 55) which could explain the variation among the authors previously cited.

On the structural nature of the polar filament; however, the results of the present study indicate a structure which differs markedly from that reported by
Schotylseck and Danneel, who described it as resembling cilia and flagella. On the contrary, Figures 54 and 55 (of a mature spore), 42, 46, 48, 49, and 57 (of sporonts with a polar filament developing) illustrate a structure that is tubular. The tubular wall may be composed of fibers but the central material does not fill the "tube" and does not represent a 9 + 2 cilium or flagellum arrangement, rather it too appears tubular (Figs. 38b, 41, and 43).

The anterior end (the end opposite that containing the coils of the polar filament (Huger, 1960)) contains an elaborately laminated structure (Fig. 54) referred to in the literature as a polaroplast (Huger, 1960). This structure begins to develop the same time that filament formation occurs (Figs. 44, 45, and 47). The formation of the polar cap also begins during sporogony and can be seen before the heavy spore coat is formed (Fig. 44). Figure 54 illustrates the cap after the spore coat is formed.

The "posterior vacuole" (Lom and Vavra, 1963a) is illustrated in Figure 15 of the light micrographs, and Figures 58 and 59 of the electron micrographs.

Nuclei, contrary to Schubert (1969a & b) who believes microsporidan spores do not possess nuclei, can be seen in the mature spore (Fig. 58). The spore of N. apis is binucleate and the late sporont, which stains much more clearly, is definitely binucleate (Figs. 44, 46, and 47).
In Figure 44 the rough endoplasmic reticulum has an appearance which is different from that seen in earlier stages. It is as though the ribosomes are much more tightly packed around the endoplasmic reticulum. Figures 54, 55, and 60 are spores cut longitudinally, in which these structures are clearly evident and Figure 61 is a micrograph of three spores cut in cross section, all containing these structures. The spore labeled "a" is a cross-section through the coils of the polar filament. The spores labeled "b" and "c" are sections cut above the coil, which is in the posterior half of the spore. Both of these sections contain a single cross-section of the polar filament indicating that the sections were cut in the region where the filament lays parallel to the longitudinal axis of the spore to the anterior end where the filament is attached to the polar cap and polaroplast.

The above mentioned micrographs give strong evidence to indicate that these structures are on sheets or tubes—not single strands—which would have to be the case to conform with Schubert who says they represent DNA helices (Schubert, 1969b).
DISCUSSION

Terminology

Before actually discussing the life cycle of *N. apis* a few problems with terminology must be considered.

Classically the diplokaryon stage is a very important stage in the life cycle of the microsporidia. It is the stage that separates schizogony from sporogony. Weiser (1961) defines this stage as "a kind of sexual process .... autogametes."

"Diplokarya (after Debaisicux, 1919) represent stages with two nuclei characterized for a period by a specific relation between these nuclei. They are sexually different parts of the same cell, a kind of pronuclei, which come into fusion during further development. This happens when the nuclei lying close together start to get blisterous and loosen, their chromatin becomes granulous and the nuclei finally melt into one another. Next, well-developed chromosomes form in the syncharyon. We find normally proceeding mitosis here, with thin, hooked chromosomes.

Their number changes from species to species between 6 and 12, and they are not easily counted. We distinguished 12 chromosomes in *Plistophora chironomi*, and we found 8-10 in *Thelohania opacta*. The fission of the nucleus yields two daughter nuclei, which contain half of the chromosomes in the pronuclei. During the formation of both stable nuclei, the separation of the plasma starts in both daughter stages simultaneously, which is the start of sporogony. We find in some genera, like that of *Octospora*, mostly two-nucleate stages till the sporogony."

Vavra (1965) in his paper on "An Electron Microscopic Study of the Morphology and the Development of some
Microsporidia", had this to say about the diplocaryon in

**Thelohania** and **Plistophora**:

"Two nuclei, very closely joined side by side ("diplocaryon") are often encountered both in the pansporoblast and in the sporoblast. But each nucleus retains its own double membrane and the phenomenon of nuclear fusion (autogamy of the classic authors) has never been put in evidence in our observations. In the pansporoblasts all the nuclei have the same fine structure and are equipotent: each nucleus gives birth to one spore. A purely "somatic" nucleus does not exist in the pansporoblast."

A two part question may be posed. Is the definition of "diplocaryon" (a) the coming together of two nuclei resulting in the fusion and formation of a single nucleus, the fission of which results in a reduction division; or (b) is the coming together of two nuclei to the point that they are closely adjacent and apparently joined but both maintaining their own integrity to be interpreted as the diplocaryon?

If we accept (a), is it a necessary part of every microsporidan life cycle? Careful examination of the literature indicates that most authors have taken the occurrence of this stage on blind faith. Some discuss only the stages before and after it stating they did not observe it. Other authors discuss schizogony and say "after the diplocaryon stage ..." implying the possibility of having seen it but not giving any description of that stage while describing all the others. If we take (b) given above, the question becomes; does it necessarily imply sexuality? Many authors
have demonstrated this structure (e.g., Vavra, 1965) from which the above quote was taken, and Sprague and Vernik (1969a) in which they posed a similar question. In the present study, nuclei have been observed in the "closely adjacent" condition throughout all the phases of development of the parasite (Figs. 25, 26, and 27). Does this mean that all these early stages are stages of sporogony because the "diplocaryon" structure is present? The complexity of the problem thus increases. Now other facets of the problem arise: How then do we define sporogony? Does it mean all stages following the 1st diplocaryon or does it mean only the stages involved in spore formation? If there is nuclear interaction in the long chain structure (Fig. 24) can this properly be called schizogony since schizogony refers to asexual reproduction? The definition of the genus Nosema is, one sporont gives rise to one spore and a sporont is the cell resulting from the diplocaryon. By definition (b) given above, Figure 24 is an illustration of a diplocaryon and in N. apis there are several cell divisions after this stage, i.e. after sporogony commences. By this reasoning N. apis does not belong in the genus Nosema.

The above mentioned situation, although in dire need of attention, is not at issue in the present study. It is only mentioned to point up the need for a clear demonstration of forms in morphogenesis and the rethinking of traditional concepts for taxon designation based on early inadequate
information.

For the sake of clarity in discussing the stages of development in the life cycle of *N. apis* the following system will be employed:

The life cycle will be divided into two phases: (a) **the proliferative phase** - including all stages from the sporoplasm extruded from the polar filament through the last cell division; and (b) **the sporogonic phase** - from the last cell division to the formation of the completed spore, or simply spore formation.

The light and electron microscopic results will be discussed concurrently as they both contribute to the understanding and interpretation of the independent results of each of the two methods in defining the morphogenesis of the parasite.

**Life Cycle**

The Proliferative Phase:

The sporoplasm as seen in the cytoplasm of the host cells (Figures 2 and 3 (light micrographs) and Figures 19 and 20 (electron micrographs)) is a binucleate cell with a thin limiting membrane surrounding it. Paired nuclei are present in all phases of the life cycle. Rough endoplasmic reticulum and free ribosomes seem to be the only cytoplasmic organelles present in this stage of development.
As development proceeds, the cytoplasm becomes more complex in makeup. Rough endoplasmic reticulum becomes more abundant (Fig. 29), the cytoplasm becomes more vesicular, and the nuclei undergo many divisions. At the same time, the cell elongates, and its limiting membrane forms indentations around each pair of nuclei (Figs. 6 and 24). The indentations deepen until the elongate structure divides into several binucleate cells (Fig. 7). It is in this phase of development that the cell limiting membrane begins to thicken. The membrane thickens in patches on the outside of the original thin limiting membrane (Figs. 30, 31, and 32). Eventually the entire membrane is thickened. Nuclear division continues occurring after this membrane thickening process has occurred (Fig. 35). These cells divide again so that the resulting cell is binucleate. The rough endoplasmic reticulum is very prevalent in this and the stages which follow. A structure not previously present in the cells develops at this stage. This structure can best be described as a complexly branched vesicular sphere (Fig. 37). Since it is demonstrated in this study that the vesicular complex is associated with the formation of the polar filament and related structures, the stages from the formation of this vesicular complex to completion at spore formation is designated to be sporogonic phase.
Sporogonic Phase:

Polar filament formation has been discussed in the literature by a few authors: Sprague, Vernick, and Vavra. Sprague and Vernick (1968b) suggest that the polar filament evolves from a nuclear spindle primordia in Glugea. The basal attachment of the filament is formed during the last cell and nuclear division by the cell pinching into two parts while the spindle fibers of the two nuclei are still connected. The nuclear spindle fibers, along with the nuclear membrane surrounding them, elongate and form the coils of the filament.

The micrographs of Nosema apis in this study do not support the above hypothesis. A sporont containing the filament basal attachment and the paired rounded nuclei in interphase next to it with a cross section of the polar filament in the upper right hand corner is shown in Figure 47. Figure 44 is a micrograph of a sporont again with the polarcap-polaroplast-filament attachment complex located on the same end of the cell with a pair of nuclei and the polar filament coils located in the opposite end of the cell. In addition, all the micrographs of N. apis examined in this study contained a complex vesicular structure along with the developing polar filament. Figure 38 is a micrograph of such a vesicular structure attached to the polaroplast and Figures 50 and 42 are micrographs of it in the center of the coils of the developing filament. Bowers and Korn (1969)
illustrate this type of a structure in their electron micrographs of Acanthamoeba castellanii (protozoa) the structure is referred to as a Golgi complex. Ito (1962) illustrates a Golgi complex in rat liver similar to this; and Vavra (1965) saw similar vesicles in Thelohania bracteata and Plistophora debaisieuxi (microsporid) which he identified as Golgi vesicles. Vavra (1965) suggests that the Golgi coalesces to form the polar filament. In the micrographs of N. apis illustrated in this work, Vavra's hypothesis is strongly supported. Microtubules, as well as Golgi, may be involved in the formation of the polar filament as they are sometimes observed in conjunction with the Golgi complex (Fig. 49). The present study may not differ with Sprague and Vernick as much as it first appears because the spindle fibers of Glugea werssenbergi and the microtubules of N. apis could be homologous. In N. apis the last nuclear division is followed by several cellular changes in which the spindle fibers are no longer observable. It has been shown, however, (Mazia, 1968) that spindle fibers are actually microtubules and Ledbetter (1968) in his paper on "The disposition of microtubules in plant cells" shows that it is the cytoplasmic microtubules which are involved in the formation of the spindle during mitosis. These papers, along with Vavra's (1965) and Sprague and Vernick's (1968b) explanation of the polar filament formation, and the micrographs shown in this study all tie together to give strong implications that the
microtubules and the Golgi complex found in the cytoplasm of the sporont contribute to the formation of the polar filament.

It is possible that the filament formation initially starts with the formation of a large round vesicle with an osmophilic periphery attached to the Golgi complex (Figs. 37 and 38b). A dark 'caplike' structure develops on the inner periphery of this vesicle (Fig. 39) and it is the start of the polar cap development. The polar filament then is formed as an outgrowth of this basal attachment. The Golgi complex which contributes to the filaments formation and elongation is attached to it. As the filament elongation proceeds, the Golgi complex becomes located at the opposite end of the cell and the numerous coils of the filament are formed around it.

The development of the internal structure of the polar filament is illustrated in Figure 39, which is a section through the developing polaroplast and polar cap. The most osmophilic area representing the primordia of the polar cap. The 'dark material', seen in the center or off center in all cross sections of the developing polar filament, is seen attached to the polar cap. It is possible that this 'dark material' represents a tube which thus becomes anchored to the anterior most portion of the internal structure of the mature spore, the polar cap, and extends the length of the filament. It is present in all sections through the
developing filament; Figs. 45, 46, 48, 49, 50, etc.. Vavra (1968) in his study of *Caudospora simulii* demonstrates this structure and refers to it as an "electron dense axis".

"Another typical feature" (of *C. simulii") "is the abnormally developed outer layer of the polar filament, while its electron dense axis seems to be very compressed. Although the polar filaments of all microsporidia examined under the electron microscope are, in fact, composed of the same number of layers in the same arrangement (Vavra, Joyon, de Putoric, 1966) there are certain differences in the individual species . . . . ".

"The internal electron dense part of the filament seems to penetrate the anchoring mass."

(the anchoring structure of the filament is the polar cap-polaroplast complex (Lom and Corliss, 1967)) "On transverse or nearly transverse sections through the terminal part of the filament axis, deeply embedded in the above mentioned granular mass, . . . . " (Vavra, 1968)

In his micrographs of the mature spore this "dense axis" appears hollow in cross section.

The structure of the polar filament has been discussed in the literature by many researchers. As indicated previously, there are two opposing attitudes as to the interpretations of this structure: some researchers believe it to be solid (Hall, 1952; Krieg, 1955; Huger, 1960; Canning, 1962; Kudo and Daniels, 1963; and Schubert, 1969a & b); and others believe that it is a tubular structure (R. Organthaler, 1922; Ohshima, 1966; Lom and Corliss, 1967; Vavra, 1965; Kramer, 1960; Ishihara, 1968; Sprague and Vernick, 1968a; Erickson, et al., 1968). Micrographs of the sporonts in which polar filament formation has already commenced, but the thick spore wall has not yet developed, are illustrated
in this study. The micrographs of the filament in the sporont are more clearly visible than in the spore stage. Figures such as 40, 42, 43, 50, 52, and 57 make it difficult to interpret the filament as anything but a tube, the contents of which has been previously discussed. Figures 43 and 50 illustrate longitudinal cuts through the filament and it is not likely to be construed as being a solid fibrilar mass such as that found in cilia or flagella as suggested by Scholtyscek and Danneel (1962) or just a solid rod. Figures 44 and 72 are micrographs of sections through enlarged portions of the filament. It is difficult to interpret a solid rod as stretching or varying in diameter to such an extent. These micrographs also demonstrate the feasibility of the tube expanding during eversion, to the degree that the sporoplasm could pass through it.

Figure 71 is a stereoscan micrograph of a spore of *N. apis* with the polar filament extruded. It is clear that this structure is the result of an eversion.

The ultra structure of the mature spore of *N. apis* corresponds with the basic pattern revealed by other authors in various species of Nosematidae (Huger, 1960, Popa, et al., 1965; Vavra, 1964). The spore shell is composed of 3 basic layers, a thin electron dense outer layer, a thick electron transparent layer, and another thin electron dense layer which surrounds the contents of the spore.
The spore coat is uniform in thickness with the exception of the polaroplast area where it is quite thin (Fig. 59).

**Nutrition**

The formation of the thickened outer cell membrane is probably more significant in relation to nutrition than to schizogony vs. sporogony. The early stages of the parasite, from the sporoplasm to the stages with the thickened cell membrane will be considered in relation to nutrition. Some sections through the long chain proliferative stage reveal the presence of minute tubular extensions (Fig. 67). In these early stages a ring of host ribosomes can be observed (Figs. 62, 63, and 64). They could be construed to be there by chance as the parasite develops, pushing the host cytoplasmic material out of its normal location, however this would make it difficult to explain why the 'ring of ribosomes' is not observed once the thickened membrane is formed around the parasite (Figs. 35, 40, 41, 43, 45, 46, etc.). Figures 32, 62, and 64 are higher magnification micrographs of the cell membrane surrounded by the ribosomes. Several bridges can be seen between the host ribosomes and the microsporidan cell membrane.

Ishihara's (1968) paper on the sporoplasm of *N. bombycis* contains several micrographs of the sporoplasm in the host
cytoplasm; a ring of ribosomes can be seen in every micrograph. In one instance he made reference to them as "Ribosomelike particles on the outer surface of the sporoplasm...." The exact nature of the relationship is not known but implications of host-parasite interaction are present. In Figures 36, 68, and 69, mitochondria are seen in very close association with the microsporidan, too close to be a passing association.

**Organelles:**

Before discussing the individual organelles it would be valuable to the understanding of this organism to discuss the cytoplasm and its constituent organelles as one living unit. It is also important that the reader consider the fact that the classic names, morphology and functions of the various organelles are the products of man's efforts to organize and categorize the activities in this milieu. It has been said many times that cells, whether they be protozoan or in man, still are basically the same. The similarities are always stressed in cell biology.

A problem arises however; the majority of electron microscopy has been done on mammals and in most instances it is from the studies on mammalian cells that the detailed analysis of the organelles have been made. Although the basic processes in cells may be the same, the means to these ends might assuredly show signs of evolution, like the
organisms that house them, and consequently differ. Example: Acid phosphatase is found in lysosomes, and lysosomes are believed to be a product of the Golgi (Novikoff, et al., 1964). Elliot (1965) worked with *Tetrahymena pyriformis* and showed that "acid phosphatase positive particles bud off as terminal dialations from the rough surfaced endoplasmic reticulum." It might be pointed out that *T. pyriformis* and many other ciliated protozoa have never had Golgi reported in them. This can be viewed as strong evidence in support of the origin of Golgi.

A second important point is that the organelles should not be thought of as separate and distinct entities. More and more researchers are finding, as they examine cells by electron microscopy that there are no real lines of demarcation, rather a continuum from one organelle to another. Example: "Like the smooth endoplasmic reticulum, the elements of the Golgi apparatus are sacs enclosed by a 60Å unit membrane and they lack attached ribosomes; several authors, in fact, have detected apparent continuities between the Golgi membranes and the membranes of the E.R., and some investigators regard the Golgi complex as a differentiated part of the E.R." (Du Praw, 1968).

In other papers "continuities" have been shown between smooth and rough endoplasmic reticulum and between nuclear membranes and endoplasmic reticulum. Novikoff, et al., (1964) in a diagram of their theory of lysosome origin show
rough endoplasmic reticulum, smooth endoplasmic reticulum, Golgi, lysosomes and antophagic vacuoles as a continuum.

With the above in mind the entire cytoplasm of *N. apis* then will be characterized before discussing its organelles.

The cytoplasm, as it is first seen in the sporoplasm, appears relatively simple compared with what later develops. There are many small pieces of rough endoplasmic reticulum and the cytoplasm generally appears granular. However, as proliferation commences and proceeds, the cytoplasm changes in appearance, it becomes quite vesicular. In some areas there are many rows of rough ER; in other areas there are complex branched systems of vesicles, some smooth and some possessing ribosomes (Figs. 28, 29, and 63). One may refer to the smooth surfaced vesicles as smooth ER or Golgi. The cell's outer limiting membrane possesses many minute tubular extensions which protrude into the host cell's cytoplasm (Fig. 67).

After the elongated cell completes its division into several binucleate cells, the outer limiting membrane of each binucleate begins to thicken. This is not done uniformly but rather in patches and on the outside of the original membrane (Fig. 32). After the last cell division has occurred, a very branched, smooth-surfaced, rounded, vesicular complex forms in the cytoplasm. A large, sack-like, smooth-surfaced vesicle is seen in association with this vesicular complex as well as a round vesicle with an
extremely osmiophilic periphery (Figs. 32, 37, and 38).

After polar filament formation commences the vesicular complex is always seen in the midst of the filament coils at the other end of the cell, the filament having elongated the length of the cell already. In micrographs of longitudinal sections of sporonts, with even just three coils of the filament formed, the basal attachment of the polar filament (polar cap + polaroplast) can be seen (Figs. 45, 46, and 47).

It is a hypothesis of this study that the vesicle with the dark periphery is the primordia of the polar cap–polaroplast formation and the thin membranated vesicle does not undergo metamorphosis but remains present in the cytoplasm (Figs. 45, 47, and 52).

To briefly summarize the state of the cell at this point, there is a dense polaroplast with a denser polar cap forming at the extreme anterior end of the cell. The filament is attached to these structures and extends back to approximately the posterior 1/3 of the cell where it coils around the vesicular complex with which it is intimately involved. Many attachments can be seen between the vesicular complex and the developing polar filament. It is probable that the vesicular complex serves as some sort of a template for filament formation (Figs. 42, 43, and 49). Attachments can also be seen between the large vesicular sacs in the extreme posterior region of the cell and the
vesicular complex (Figs. 43 and 49).

It is a contention of this study that all these structures are integrated parts of each other and to try to discuss each as an independent structure without discussing their relationships to each other would not adequately describe the system.

The vesicular sacs and vesicular complex could most probably be called Golgi. Bowers and Korn (1969) refer to a similar structure in Acanthamoeba as Golgi, and it appears quite probable that it is functioning in a secretory capacity.

The rough endoplasmic reticulum is abundant at this stage of development (Figs. 46, 47, and 51). From these observations it is tentatively concluded that this material forms poly-ribosomes in the spore stage (Figs. 54, 55, 60, and 61). These structures have been described in many organisms, from vertebrate muscle (Spiro and Hagopian, 1968) to bacteria, in E. coli (Kingsbury and Voelz, 1969). Schubert (1969a) believes that the nuclei break down and that these structures represent DNA helices. However, two nuclei are observed in the sporont (Fig. 41), a more developed sporont, with many polar filament coils (Fig. 44, note also the rough ER in this cell), and two nuclei are observed in the spore (Fig. 58). It might also be pointed out that, in Ishara's study of the sporoplasm of N. bombicies, the sporoplasm, immediately after extrusion from the polar filament,
contained two nuclei. In fact, in *N. apis*, paired nuclei characterize all the phases of development from sporoplasm (Figs. 2 and 3) through the proliferating stages (Fig. 24, and enlargements from it) through spore formation (Fig. 41) and the mature spore (Fig. 68).

Whether or not exchange of nuclear material is occurring between these paired nuclei during this association is a point well worth investigating. Figures 30 and 70 seem to present this possibility.

Nuclear division occurs by the formation of a spindle within the nuclear membrane without it ever breaking down. This phenomenon has been reported for other microsporidia (Vavra, 1965; Sprague and Vernick, 1968b), other protozoa (Cleveland, 1963), and in yeast (Moor, 1967). Figures 28, 35, and 36 illustrate this phenomenon. The nuclear membrane remains intact throughout the division process (Fig. 36 is in Metaphase). Figure 35 is a micrograph of a nucleus in telaphase, the nucleus is "dumbbell" shaped and the membrane is still intact.

No lysosomes were observed nor have they been reported in the microsporidia studied thus far.

A concentric membraned or laminated structure has been observed in many sections (Figs. 33, 41, 47, 48, and 57), although mitochondria as we recognize them structurally have not been observed in microsporidia. Rudziaska and Trager (1959) suggest the possibility that a concentric membrane
structure such as those they observed in the malaria parasite possessed mitochondrial activity. Laminated structures such as these have also been demonstrated by Bowers and Korn (1969) in *Acanthamoeba castellanii* during encystment. These laminated structures were, in almost every case associated with the mitochondria. However, it should be noted that until some histochemical or biochemical studies can be done, no conclusions can be drawn as to the nature of this structure in the microsporida.

The literature on microsporidan ribosomes consists of one paper by Ishihara and Hayashi (1968) in which they studied the sedimentation coefficients ("S" values) of the ribosomes of *N. bombicida*. Throughout the plant and animal kingdoms ribosomal "S" values thus studied are of two types, 70S and 80S. It has been found that the 70S ribosomes are characteristic of protokaryotes (bacteria and blue-green algae). "The similarity of the sedimentation coefficients of the ribosome from all the species of bacteria and blue-green algae studied permits us to think that they are all characterized by approximately the same shape and sizes as the ribosome of *E. coli*. Thus the molecular weight of the 70S ribosomes of protokaryotic organisms is about $3 \times 10^6$, . . . . " (Spirin and Gavrilova, 1969). 70S ribosomes are also found in chloroplasts and mitochondria. "In all higher, eukaryotic, organisms, the ribosomes are contained not only among the basic mass of the cytoplasm and nucleus, but also
in the 'energy-giving' organoids of the cell, the chloroplasts and mitochondria.

It has been shown that the ribosomes of the chloroplasts of green plants differ distinctly from the cytoplasmic 80S ribosomes of the same cells: they are somewhat smaller and are of the 70S type, in which they exhibit a surprising similarity to the bacterial ribosomes." (Spirin and Gavrilova, op cit.).

With the exception of the above mentioned chloroplast and mitochondrial ribosomes, all ribosomes from eukaryotes are of the 80S type (Taylor and Storck, 1964) including protozoa other than microsporida (Whitson, et al., 1966; Prestayko and Fisher, 1966; Chesters, 1966).

With the above information in mind it is surprising to find that *N. bombicus* (a eukaryote) has ribosomes which are 70S. Their results are significant in that this is the first microsporidan paper with definite taxonomic implications.

The ribosomes in the spore, as seen in *N. apis* in the present study, have not yet been described in the literature. Shubert (1969a) illustrates these structures in the new microsporidan he describes but he suggests that the nuclei break down during spore formation and are not present in the spore. He then goes on to describe the structures present in the sporoplasm as DNA helices. It is difficult to imagine that there are nuclei present in the sporont before
spore formation and in the sporoplasm immediately after emergence from the polar filament and yet none in the spore. In addition, nuclei have been described in the spore in the present study as well as by other authors (Lom and Corliss, 1967; Lom and Vavra, 1963a; Dissanaike and Canning, 1957). It is suggested by the present study that the structures present in *N. apis* are polyribosomes, such as those described in *E. coli* (Kingsbury and Voelz, 1969) and in vertebrate muscle (Spiro and Hagopian, 1968).

With the exception of the microsporidan described by Sprague, Vernick and Lloyd (1968), in which they found 'bristle-like structures' present on the spore coat, all researchers have found essentially the same type of spore wall. Essentially it is composed of two thin electron dense areas with a thick electron transparent zone between them. Interpretations of this area have differed with different authors however. Some refer to it as one layer which stains only on the outer and inner edges; some interpret it as two layers, a thin inner dark staining area surrounded by a thick electron transparent zone which stains on the surface; and still others who interpret it as three layers, two thin and electron dense, one thick and electron transparent.
CONCLUSION

Schizogony, sporogony, and diplocaryon are terms which are used for processes and forms in the life cycle of microsporida. The present study has revealed a need to consider these terms in the light of new knowledge and possibly redefine them if they should be continued in use. In the development of *Nosema apis*, in the present study, these terms are shown to be inaccurate and not useful by their traditional definitions. Vavra (1965) and Sprague and Vernick (1969a) have expressed concern as to the definition of the "diplocaryon" in the development of representatives in the genera *Thelohania*, *Plisthopliora*, and *Nosema*.

The consideration of developmental phenomena and terminology must be done in light of information for several genera. For example, depending on the definition of the diplocaryon (a- complete nuclear fusion, or b- a "closely adjacent association", without nuclei losing their identities) *Nosema apis* either does not have a diplocaryon stage, or *N. apis* has several such stages followed by a series of cytokineses after first occurrence of the diplocaryon. Either of these situations raises a question as to the correct generic designation since the genus *Nosema* is defined to include only those microsporidians whose last cell division occurs before the diplocaryon stage.

42.
A clearly defined system of nomenclature is adopted for the present study to avoid misunderstanding of terminology by researchers using the results. All stages, from the discharged sporoplasm through the last cell division are referred to as stages in the "proliferative phase" of development. All stages of spore formation are referred to as the "sporogonic phase". The "diplocaryon" is shown in this study of *N. apis* to be accurately described as having nuclei closely adjacent without loss of identity (see "b" above). The life cycle of *N. apis* was studied with the aid of light and electron microscopy. It is evident that both techniques must be used concurrently in the study of any microsporidan, because each provides unique opportunities to study the biology of intracellular parasites. Either method by itself does not provide sufficient information for biological nomenclatorial or taxonomic considerations. For example, the sporoplasm stage appears binucleate with light microscopy (Fig. 3), yet with electron microscopy may appear mononucleate. In contrast, polar filament formation and the organelles apparently associated with the process can not be defined with light microscopy.

The present study reveals many structural entities and their development that have not heretofore been documented in the life cycle of *N. apis*. The sporoplasm, after several nuclear divisions without cytokinesis, becomes a long chain proliferative stage, similar to a stage described with light
microscopy in other species of Nosema. However, there is one very important difference which has not been reported to this time. All the nuclei were observed in pairs and in the diplocaryon form before cytokinesis when the chain-like structure produces many binucleate cells. After cytokinesis the binucleate cells appear to be typical diplocarya (definition "b" above), however, nuclear division continues to occur in Nosema apis. It can be concluded that the two stages in the maturation of the parasite during proliferate phases, that could be confused in their diplocaryon forms, may be distinguished by recognizable differences in the cytoplasmic limiting membrane thickness. Material is deposited on the outside of the thin cell membrane in patches until it encompasses the entire cell.

The first sporogonic form, which develops into a spore, is binucleate and surrounded by a thickened membrane. Based upon the morphogenic evidence presented, the vesicular complex and the saclike vesicles attached to it may function as the Golgi complex, and this Golgi complex is active in the formation of the polar filament, the polar cap and the polaroplast. Further, the "electron dense axis" of the developing polar filament in the maturing spore becomes centrally located within the filament of the mature spore. This spacial adjustment may be due to the thickening of the filament walls. Rough endoplasmic reticulum is seen in the spore as polyribosomes or very densely packed ribosomes.
The spore is binucleate and the spore coat is like that described in the literature for other microsporida.

**Perspectives**

After many representative types of microsporida are studied, the classification and terminology need to be reviewed in light of new information, similar to that developed in this study of *N. apis*. Histochemical investigations need to be pursued to detect the function and nature of structures in the developing parasite.

Research needs to be done with the parasite in tissue culture so the parasite can be observed *in situ* under controlled conditions.
SUMMARY

*Nosema apis* infects ventricular epithelial cells of the adult honey bee, *Apis mellifera*. The bees can be infected by incorporating spores of the parasite in their food.

Adult worker honey bees, newly emerged, were marked and placed in either of two miniature observation hives (one experimental and one control) and kept in an incubator at 31°C for three days to mature. Combs of unsealed brood were added to each hive enabling the marked bees to perform normally as nurse bees.

When the marked bees were seven days old, the bees in one hive were fed parasite spore inoculum in the sugar water feeding bottle (3 x 10⁷ spores/ml), while uncontaminated sugar water was given to the bees in the control hive. *N. apis* spores extrude their polar filaments after gaining access to the honey bee ventriculus. It is a generally accepted opinion, though not clearly demonstrated, that the sporoplasm is "injected" into the host gut cell. The binucleate sporoplasm, once inside the host cell, begins to proliferate and it forms minute tubular extensions from its periphery which may aid in the nutrition of the parasite during its development. The nuclear membrane never breaks down throughout karyokinesis, the cell elongates and the nuclei form a series of pairs which after cytokinesis result in many binucleate cells.
While the proliferation process is taking place the cytoplasm of the parasite becomes more and more vesicular in appearance. The binucleate stages gradually acquire a thickened cell limiting membrane by deposition of material on the outside of the former thin limiting membrane.

Nuclear division continues, accompanied by cell division, the final division resulting in binucleate cells each with a thick, limiting membrane.

A vesicular complex develops in the cytoplasm, with two sack-like structures attached to it. One has a thin membrane and is irregular in shape, and other is spherical and develops an extremely osmiophilic periphery. It is probable that the latter structure is the beginning of the polar cap-polaroplast complex, because the developing polar cap-polaroplast is seen in cells of later stages and is located in the same area as this structure. The developing structure is not very different morphologically from what was attached to the vesicular complex earlier. The vesicular complex is attached to the round vesicle with the dark periphery first, then it is attached to the developing polar cap polaroplast complex. As development proceeds increasing numbers of polar filament coils develop. At the extreme posterior end of the cell are several large smooth membraned sacs. These sacs appear to be attached to the vesicular complex and it is suggested they be called Golgi because of their association with polar filament formation.
In the spore stage the filament is seen with about 20-30 coils found in one end of the spore, a pair of nuclei in the opposing half, and a very membranous polaroplast with a polar cap and the polar filament attachment in the anterior tip of the spore. Ribosomes are seen in an array that appears to be the pattern resulting from their becoming very tightly packed. The "posterior vacuole" can be clearly seen in the light micrographs and appears very laminated in the electron micrographs, similar to the polaroplast.

The spore coat has the classically described two electron dense thin areas located on each side of a electron transparent area. The spore coat is fairly uniform in thickness with the exception of the area immediately anterior to the polaroplast. The electron transparent portion of the spore coat is greatly reduced in diameter at this point.

This study revealed the need for reinvestigation of many microsporidan life cycles incorporating both light and electron microscopy to determine the life cycles in detail. Light microscopy, although informative and necessary, is inadequate by itself. Light microscopy does not reveal that the nuclei are in paired "diplocaryon" form throughout proliferative development. The development process of the spore and its structures cannot be observed by light microscopy alone.
The study revealed a need to redefine classic terms in light of new information on the structures observed. For example, the definition of the diplocaryon, and the beginning of sporogony by classical light microscopy interpretations have influenced the definitions of genera in the Order Microsporida, depending on how these terms are defined.
ABBREVIATIONS USED ON FIGURES

C = cytoplasm of N. apis.
CE = centriole.
CM = concentric membrane structure.
CR = chromosomes.
D = "diplocaryon nuclei" of N. apis (see p. 23, definition "b").
DA = dense axis of polar filament.
HC = host ventricular cell.
HN = host nucleus.
HR = host ribosomes.
IC = inner, thin, electron dense area of spore coat.
LPS = long chain proliferate stage.
M = host cell mitochondrion.
MT = microtubules.
MV = host cell microvilli.
N = nucleus of N. apis.
NM = nuclear membrane.
NO = nucleolus.
OC = outer, thin, electron dense area of spore coat.
P = polaroplast.
PC = polar cap.
PF = polar filament.
PP = polaroplast primordia.
PS = proliferate stages.
PV = posterior vacuole.
R = ribosomes or polyribosomes.
RER = rough endoplasmic reticulum.
S = smooth surfaced endoplasmic reticulum.
SA = spindle aster.
SF = spindle fibers.
SP = sporoplasm.
SS = smooth surfaced, sac-like vesicle complex.
SV = smooth surfaced, sac-like single large vesicle.
TC = thick, electron transparent area of spore coat.
TE = tubular extensions.
TK = thick cell membrane.
TN = thin cell membrane.
UHC = uninfected ventricular epithelial cell.
VC = vesicular complex.
* = vesicular complex interaction with polar filament formation.
Figures 1-9

Infection of Apis mellifera ventricular (midgut) cells with Nosema apis. 1. Binucleate planont at end of polar filament. 2-3. Binucleate planonts implanted in cytoplasm of host ventricular cells. 4. Beginning of first nuclear division, cell has elongated. 5. First nuclear division, four nuclei forming in elongated cell. 6. Long chain of binucleates, formed after many nuclear divisions. 7. Binucleate schizonts after long chain has divided into many binucleate cells. 8. Beginning of second schizogonic chain. 9. Second schizogonic chain with six chromatin-rich nuclei. 1000 X Fig. 1; 2000 X Figs. 2-9.
FIGURES 1-9
Figures 10-15

Figure 16

Section of host (*A. melifera*) ventricular epithelial cells, some uninfected and some containing binucleate sporoplasm stages of *N. apis* in their cytoplasm. 1,100 X.

Figure 17

Section of host ventricular epithelial cells containing many proliferate stages of *N. apis*, some are long chain multinucleates and some are binucleates. 1,100 X.
FIGURES 16-17
Figure 18

An entire epithelial cell, as viewed on a phase contrast microscope, heavily burdened with *N. apis* spores. Note the nucleus is uninfected. 1,100 X.
Figure 19

*N. apis* sporoplasm located in the cytoplasm of a ventricular epithelial cell. 11,100 X.
Figure 20

*N. apis* sporoplasm located in the cytoplasm of a ventricular epithelial cell. Note host mitochondria in very close association with the sporoplasm's limiting membrane. Rough endoplasmic reticulum with ribosomes tightly packed on membranes also present. 22,200 X.
Figure 21

Section of host ventricular epithelial cell containing proliferate chain of *N. apis*. Host microvilli in cross section. 11,100.
Figure 22.

Detail of Figure 21. 22,200 X.
Figure 23

Additional section through host cell shown in Figure 21 to provide evidence of *N. apis* proliferative cell morphology. 11,100 X.
FIGURE 23
Figure 24

Section of host ventricular epithelial cell containing longitudinal section through a large portion of a long chain proliferate stage. Numbers 1-3 indicate portions of this micrograph illustrated in detail in the following three figures. 8,300 X.
Figure 25

Detail of area 1 in Figure 24. Note the presence of two diplocarya in one cell. 22,200 X.
Figure 26

Detail of area 2 in Figure 24. 22,200 X.
FIGURE 26
Figure 27

Detail of area 3 in Figure 24. 22,200 X.
Figure 28

Section of proliferate cell with a diplocaryon containing a spindle aster in the periphery of one of the nuclei. 44,800 X.
Figure 29

Section of proliferate stage with a vesicular cytoplasm.  
44,800 X.
Figure 30

Diplocaryon proliferate form of *N. apis*. Note abundant rough endoplasmic reticulum, possible transfer of nuclear material (encircled) and thickening of parasite limiting membrane. 44,800 X.
FIGURE 30
Figure 31

Proliferate forms of *N. apis* with apparent irregular distribution of thick areas of limiting membrane. 44,800 X.
Figure 32

Detail of limiting membrane possessing some thick areas and some thin. Note the thickening is formed as deposits of material on the outside of the thin membrane. Note also the connections between the thin membrane and the host ribosomes. 142,000 X.
Figure 33

Section of host ventricular epithelial cell containing parasite cells with entire limiting membrane thickened. In cell "a" note the presence of vesicular complex and thin membraned sac. Cell "b" contains a concentric membraned structure. 30,000 X.
Figure 34

Section through proliferate stage with entire limiting membrane thickened. Note the tubular endoplasmic reticulum. 44,800 X.
Figure 35

Section through 2nd elongate proliferate stage. Note the thick membrane surrounding this cell and the diplocaryon with one nucleus in anaphase (chromosomes are located near the base of each aster). The overlay outlines the nuclear membranes, spindle fibers can also be seen. 20,600 X.
"a", "b", and "c" are sections of cells possessing dividing diplocarya, note that all 3 cells possess thickened cell membranes. Cell "a" possesses a nucleus which appears to be in metaphase. In the insert of part of cell "b" the nuclear membranes are clearly visible and a spindle is located at the base of one of the diplocaryon nuclei. Cell "c" is a cross section through a dividing nucleus, note the spindle fibers, chromosomes, and double nuclear membrane. Cell "a" and "c" 23,000 X; cell "b" 46,000 X.
Figure 37

Section through a ventricular epithelial cell containing several *N. apis* cells, most of which are early stages of the sporogonic phase. Cell "a" contains the rounded vesicle with the dark periphery attached to the vesicular complex. Cell "b" contains the thin membraned sac-like structure as well as what appears to be the beginning of the vesicle with a dark periphery both attached to the vesicular complex. 14,600 X.
Figure 38

Sections through 4 cells containing different views of *N. apis* sporont cells in the same stage of development as Figure 37. Cells "a", "b", and "d" 18,400 X; cell "c" 24,900 X.
Figure 39

Section through a ventricular epithelial cell containing a sporont stage of *N. apis*. This cell is further developed than those in previous figures. Note the round vesicle with the dark periphery. The dark periphery has greatly increased in diameter (polaroplast primordia) and an even darker area (polar cap primordia) can be seen with the "dense axis" of the filament attached to it. Filament elongation has commenced by this time. Note the 2 cross sections of polar filament present in the cytoplasm and that the vesicular complex is no longer present in this area. 24,900 X.
Figure 40

Section through ventricular epithelial cell containing cross section *N. apis* sporont with at least two coils of the polar filament present around the vesicular complex. 45,800 X.
FIGURE 40
Figure 41

Section through ventricular epithelial cell containing longitudinal section of *N. apis* sporont with 3 coils of polar filament present around vesicular complex. Note the presence of diplocaryon and both nuclei are in interphase. A concentric membraned or laminated structure is also present in this section. 32,200 X.
Figure 42

Tangential section through area of developing polar filament. Many coils have formed around vesicular complex. Note electron dense axis is present in every section of polar filament. Note the interconnections between the developing polar filament and the vesicular complex. 39,400 X.
FIGURE 42
Figure 43

Tangenital section through sporogonic phase of *N. apis*. The large saclike dense vesicles are present in this section. Note their intimate association with the vesicular complex. 45,800 X.
FIGURE 43
Figure 44

Longitudinal section of *N. apis* sporont. Note the relative locations of organelles from anterior end (anterior-posterior area designations established by Huger, 1960) with the polar cap-polaroplast primortia and attached polar filament, two interphase nuclei present and rough endoplasmic reticulum possessing very tightly packed ribosomes. 25,900 X.
FIGURE 44
Figure 45

Longitudinal section of *N. apis* cell in sporogonic phase showing the relative locations of cell organelles. 25,700 X.
Figure 46

Longitudinal section through posterior end of *N. apis* sporont showing detail of organelles. 32,200 X.
Figure 47

Longitudinal section through anterior end of same cell illustrated in Figure 46. Note nuclei are seen in diplocaryon form; concentric membraned structure present and large thin membraned sac seen as extension of rough endoplasmic reticulum. 32,200 X.
Figure 48

Longitudinal section through sporont stage. Note the abundance and nature of the rough endoplasmic reticulum. 39,400 X.
Figure 49

Longitudinal section through posterior end of sporont. Note the connections between the many sections of polar filament and between the polar filament and the vesicular complex. Note also the presence of microtubules. 56,200 X.
Figure 50

Cross section through vesicular complex and developing polar filament. Note the many connections between both structures. 45,800 X.
Figure 51

Cross section through mid region of sporont. Note the single cross section of polar filament indicating histological section was cut through area of sporont where polar filament lays parallel to longitudinal axis of sporont. Note also the abundance and nature of the rough endoplasmic reticulum it is lamellar and ribosomes are abundant. 32,200 X.
Figure 52

Cross section (cell "a") and tangential section (cell "b") of two sporonts. 24,900 X.
Section through *N. apis* mature spore. Note the different spore coat areas. 67,100 X.
FIGURE 53
Figure 54

Longitudinal section through anterior end of mature spore. Note the matured forms of the heretofore developing structures. 67,100 X.
Figure 55

Longitudinal section through posterior end of spore pictured in Figure 54. Note the ribosomes and polar filament. 67,000 X.
Figure 56

Tangenital sections through mature spores. 26,400 X.
Figure 57

Section through small portion of sporont with developing polar filament. 92,900 X.
Figure 58

Longitudinal section through mature spore showing the relative location of structures. Note the diplocaryon nuclei. 26,400 X.
Figure 59

Longitudinal section through a mature spore which was probably about to discharge its polar filament. Note the extended polaroplast and posterior vacuole, it appears as though they could possibly be connected. Note also the shape of the spore coat and the thinner electron transparent area where polar filament extrusion occurs. 29,200 X.
Figure 60

Tangentlal section through mature spore. Note the ribosomes in area "a" and area "b" indicating that they are most probably in a long tubular array very similar to the rough endoplasmic reticulum of earlier stages. 39,400 X.
Figure 61

Cross section through three mature spores ("a", "b", and "c"). Spore section "a" is through the polar filament coil area. Spore sections "b" and "c" are from the area between the filament coils and the filament basal attachment. Note the single cross section of polar filament and the ribosomal patterns. 24,900 X.
Figure 62

Section through ventricular epithelial cell containing the edge of a thin membraned proliferate form. Note the host ribosomes in close proximity to the limiting membrane and the possible interconnections between the two. 56,200 X.
Figure 63

Cross section of proliferate stage showing proximity of host ribosomes to parasite limiting all around *N. apis* cell. Area "a" enlarged in Figure 64. 39,400 X.
Figure 64

Detail of part "a" Figure 63. Note possible interconnection between ribosomes and *N. apis* limiting membrane. 66,900 X.
Figure 65

Section through ventricular epithelial cell containing early proliferate stage. Note the ring of host ribosomes around proliferate stage and the tubular extensions from the *N. apis* cell limiting membrane. 24,900 X.
Figure 66

Section through proliferate stage of *N. apis*. Rough endoplasmic reticulum is abundant and "ring" of host ribosomes is present around parasite cell. 45,000 X.
Figure 67

Section through proliferate stage. Note the tubular extensions of *N. apis* limiting membrane. 24,900 X.
Figure 68

Detail of Figure 22 section through proliferate stage with host mitochondria in very close proximity to *N. apis* cell limiting membrane. Note possible tubular connections between the two. 24,900 X.
Figure 69

Detail of Figure 22 section through proliferate stage with host mitochondria in very close proximity to *N. apis* cell limiting membrane. Note possible tubular connections between the two. 19,500 X.
Figure 70

Detail of a diplocaryon. Note possible exchange of nuclear material. 104,000 X.
Figure 71

Stereoscan micrograph of *N. apis* spores. Note spore "a" with extruded polar filament, and spore "b" with anterior end visible, there is a definite indentation in the spore coat which coincides with the appearance of the sectioned spore coat in Figure 59.
Figure 72

Section through late sporonts. Note the distended polar filament in these cells. 13,760 X.
Embedding Technique

**Modified Aqueous Bouin's Fixative**

Saturated picric acid in distilled water 575 ml
40% Formaldehyde 125 ml
Trichloroacetic Acid 4.5 grams

Ester Wax 1960 from the Gallard-Schlesinger Chemical Manufacturing Corporation was used as the embedding medium.

1. Fix in Modified aqueous Bouin's fixative 24 hours to 1 week
2. Store in 70% ethanol - indefinitely
3. 95% ethanol - 2 hours
4. 100% ethanol - 1 hour
5. 100% methanol - 1 hour
6. 1:1 mixture of 100% methanol and methyl cellosolve - 4 hours
7. 1:1 mixture of #6 and ester wax 1960 - 4 hours to overnight
8. Fresh ester wax, 2 changes - 4 to 6 hours each
9. Embed in fresh ester wax

Steps 7 through 9 were carried out in a vacuum oven to minimize air bubbles in tissue. The tissues were embedded in paper or metal boats containing fresh ester wax and allowed to harden in the refrigerator for about 1 hour. The blocks were trimmed and then sectioned at 4 μ on an American Optical Spencer 820 Microtome with A.O. Spencer Knives which were sharpened on an A.O. Spencer 935 Automatic Microtome Knife Sharpener.

Mounting Technique

**Mounting Solution**

Mayers' Albumin Fixative 5 drops
Distilled Water 20 ml

**Glass Slides**

Cleaned glass slides were submerged in an acid alcohol solution until needed for mounting sections.

**Procedure**

Glass slides were placed on a slide warmer set at approximately 40°C. Several drops of the mounting solution were placed on each slide and the tissue
ribbons were floated on. When the sections expanded sufficiently the mounting solution was poured off and the slides were left on the warmer until they dried.

Heidenhain's Haematoxylin Stain Technique (Gurr 1962)

Staining Solutions

#1. Dissolve 4 gm of Iron alum in 100 ml of distilled water; this solution is the alum mordent.
#2. A 2% solution of Iron alum is used as a destainer.
#3. Dissolve Iron Haematoxylin in absolute alcohol to make a 10% (w/v) stock solution and allow this solution to ripen for 3 to 6 months. Staining solution: 5 ml stock solution and 95 ml distilled water

Staining Procedure

1. Remove paraffin in xylene - 2 changes, 3-5 minutes each
2. 100% ethanol - 2 changes, 3 minutes each
3. Hydrate to distilled water
4. Mordent (solution #1) 24 hours
5. Quick rinse in distilled water
6. Staining solution (#3) 24 hours
7. Quick rinse in distilled water
8. Destainer - use microscope to check destain until sections reach the desired color
9. Rinse in distilled water
10. Dehydrate
11. Clear in xylene
12. Mount in piccolyte (60% piccolyte in xylene, w/v
Electron Microscopy Technique

I. Cut tissues into 1 mm square pieces.


0.2 M Monobasic Sodium Phosphate - 2.76 gm in distilled H₂O to total 100 ml volume.

0.2 M Dibasic Sodium Phosphate - 7.17 gm in distilled H₂O to total 100 ml volume.

\[\begin{align*}
19 \text{ ml} & \quad \text{Monobasic Sodium Phosphate} \\
81 \text{ ml} & \quad \text{Dibasic Sodium Phosphate} \\
100 \text{ ml} & \\
+ 60 \text{ ml} & \quad \text{Distilled Water} \\
160 \text{ ml} & \\
+ 40 \text{ ml} & \quad 25\% \text{ Aqueous Glutaraldehyde (from Polysciences Inc.)} \\
200 \text{ ml} & \quad \text{Fixative}
\end{align*}\]

III. Wash tissue for 1 hr in Buffer Wash.

\[\begin{align*}
.2 \text{ M Monobasic Sodium Phosphate} \\
.2 \text{ M Dibasic Sodium Phosphate} \\
19 \text{ ml} & \quad \text{Monobasic Sodium Phosphate} \\
81 \text{ ml} & \quad \text{Dibasic Sodium Phosphate} \\
100 \text{ ml} & \\
+100 \text{ ml} & \quad \text{Distilled Water} \\
200 \text{ ml} & \quad \text{Buffer Wash}
\end{align*}\]

IV. Fix for 6-8 hrs with 1% OsO₄ in Phosphate Buffer. (Prepared according to Millonig, 1961. J. Appl. Phys. 32:1637.)

Solution A - 2.26% NaH₂PO₄·H₂O - 1.13 gm in 50 ml distilled water
Solution B - 2.52% NaOH - 1.26 gm in 50 ml distilled water
Solution C - 5.40% Glucose - 2.7 gm in 50 ml distilled water
Solution D - 41.5 ml Sol. A \\
\hspace{1cm} 8.5 ml Sol. B

Fixative: 45.0 ml Sol. D \\
\hspace{1cm} 5.0 ml Sol. C \\
\hspace{2cm} 0.5 gm OsO₄ crystals
V. Dehydration:

Wash tissue in distilled H₂O for five minutes.
Dehydrate tissue in:
- 35% Methyl alcohol - 5 minutes
- 50% Methyl alcohol - 5 minutes
- 70% Methyl alcohol - 5 minutes
- 80% Methyl alcohol - 5 minutes
- 95% Methyl alcohol - 5 minutes
- Absolute Methyl alcohol - 10 minutes
- Propylene Oxide - 30 minutes

VI. Infiltration:

Epoxy Resin (modification of Luft's method 1961.
J. Biophys. Biochem. Cytol. 9:409.)

Epon 812 20 ml
Araldite (Epoxy Resin 506) 20 ml
Dodecenylsuccinic Anhydride (DDSA) 60 ml
100 ml
add 2.0 ml DMP-30 to accelerate.

Put tissues in:
- 1/3 (Luft's) Epoxy Resin: 2/3 Propylene Oxide - 1 hour
- 1/2 (Luft's) Epoxy Resin: 1/2 Propylene Oxide - 1 hour
- 2/3 (Luft's) Epoxy Resin: 1/3 Propylene Oxide - 1 hour
- Pure (Luft's) Epoxy Resin - overnight

VII. Embedding:

Next day put pure (Luft's) Epoxy Resin in capsules
and drop one piece of tissue in each capsule.
Put the capsules in a 74°C oven for 3 days. Take
the capsules out and let them sit for one hour,
then they can be trimmed and sectioned. Only
gray and silver sections were picked up.
Grids:

300 mesh bare grids or 100 mesh coated grids were used.

Coating:

Stock 4% Parlodion
4 gm parlodion in 100 ml Amyl Acetate
Keep in the dark

a) Make up a 1.75% solution of parlodion in amyl acetate
(to dilute from a 4% solution mix 5.25 ml parlodion
and 6.75 ml amyl acetate)

b) Fill a clean finger bowl with distilled water. Pass
a folded paper towel across the surface of the water
and bowl to make sure the water level is to the top
of the bowl. Drop one drop of the parlodion solution
onto the center of the water surface and allow it to
spread until the colors disappear (about one minute).
Remove the film with an applicator stick by placing
the applicator stick in the center of the film and
twisting the stick. Do this about five times.

c) Allow another drop to spread (6th one) and dry, then
arrange 20-25 grids in rows on the surface of the
film shiny side down. Cover the grid with flattened
clean aluminum foil and fold the edges of the film
around the foil. Lift away the foil with the grids
attached; place the foil (grids up) into a petri dish
with filter paper inside. Cover the dish leaving a
gap for water to evaporate and escape. Dry the grids
for at least 12 hrs before coating with carbon.

Carbon coating:

Put the grids on foil in a vacuum evaporator with the
carbon rods set in at a 45° angle to the surface of the
grids. Coat the grids very lightly. They are now ready
for use.

Staining:

1) Stain with uranyl acetate: This solution should be
made up fresh. Shake a saturated solution of uranyl
acetate in distilled water for 20 minutes -- be sure
to keep solution in the dark at all times. Filter
solution just before using. Place grid, tissue side
down, on a drop of the freshly filtered uranyl acetate
solution and stain for 1 hr.
2) Wash with distilled water about 30 seconds.

3) Stain with lead citrate (J. Cell. Biol. 1963 17:208). This solution will keep for an indefinite amount of time. When a precipitate is visible the solution is no longer useable.

   a) Place 1.2994 gm Pb(NO\textsubscript{3})\textsubscript{2}; 1.79 gm Na\textsubscript{3}(C\textsubscript{6}H\textsubscript{5}O\textsubscript{7}) \cdot 2H\textsubscript{2}O; and 30 ml water in a 50 ml volumetric flask.

   b) Shake this solution vigorously for 1 minute and allow it to stand with intermittent shaking for 30 minutes.

   c) Add 8.0 ml of 0.1N NaOH (make fresh) and dilute above solution to 50 ml. Mix by inversion. Mild turbidity may be removed by centrifugation. Filter solution just before using and do not breath on it. Stain tissue for 6 minutes.

4) Wash with 0.1N NaOH about 30 seconds.

5) Wash with distilled water about 30 seconds.
REFERENCES


