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Ultrastructural Qualitative and Quantitative Data on the Sporogenesis of the Protozoan *Abelspora portucalensis* (Microspora, Abelsporidae): A Different Approach to the Study of Microsporidia

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ABSTRACT The sporogenesis of the microsporidium *Abelspora portucalensis* was studied with electron microscopy. In qualitative terms, new aspects of the cytoplasmic ultrastructure of the schizont, sporont, and sporoblast are described: the presence of microtubules, of aggregates of small opaque vesicles, and of dispersed larger vesicles with clear matrix. The hypothesis that the opaque vesicles may represent the Golgi apparatus and the clear vesicles may correspond to the smooth endoplasmic reticulum is discussed. The use of standard stereological and statistical techniques gives us a new perspective on the development of this microsporidium. The most relevant quantitative data display that the amount of rough endoplasmic reticulum (either in relative or absolute terms) presents significant differences among the three stages, with the sporont showing the highest values; that the absolute (but not the relative) volume of the large vesicles significantly changes during sporogenesis with the highest values presented by the sporont; that the surface-to-volume ratio of the schizont and sporont cells is similar and significantly greater than that of the sporoblast cell; that the surface density of the nucleus in relation to soma remains constant in the three stages (on the contrary, the surface-to-volume ratio of the nucleus increases and its volumetric density diminishes); and finally, that the nucleolus decreases its relative and absolute volumes. The functional significance of these results is analyzed and the application of similar methodology in quantifying the effects of drugs upon microsporidia is suggested.

The Microspora is a phylum of intracellular protozoan parasites which have very small spores with a characteristic complex structure. Their study is not purely of academic interest, because of their economical importance as destructive parasites, for instance of honeybees, silkworm larvae, crabs, shrimps, and fishes (Sprague, '82) or as controlling agents against undesirable insect species (e.g., MacVean and Capinera, '91; Odindo, '90). In addition, it was noted that microsporidia may become important pathogens for immunosuppressed patients, including those with AIDS (Shadduck, '89).

The study of microsporidia dates from early in this century; taxonomic monographs were published by Auerbach ('10) and Kudo ('24). Presently, microsporidiology develops rapidly; indeed, new species and genera appear continuously and new information changes the conception of existing taxa (Larsson, '88). The early works were essentially concerned with structure, life cycles, and taxonomy, but the number of papers on cell culture (e.g., Iwano and Ishiara, '89, '91), spore germination (e.g., Undeen, '90; Undeen and Epsky, '90), and immunology (e.g., Hollister et al., '91; Ke et al., '90), among other fields of interest, is growing fast.

Despite the great deal of information concerning ultrastructural details on microsporidia, that knowledge is based on qualitative grounds. As far as we know, there is no quantitative study on the sporogenesis of any microsporidium. The immediate interest of such a study is to achieve a better understanding of the process, to obtain new data, and to avoid drawbacks inherent to qualitative observations.

The present paper offers a stereological study of the sporogenesis of *Abelspora portucalensis* (Microspora, Abelsporidae), along with a brief qualitative approach. New information about the life cycle of this microsporidium is reported, increasing the previous knowledge about the species (Azevedo, '87).

MATERIALS AND METHODS

Preparation of the material for electron microscopy

Adult specimens of *Carcinus maenas* (Crustacea, Decapoda) were caught in the salt marshes of Aveiro (North of Portugal) in March, 1991. The animals were dissected and their hepatopancreas observed for the presence of cysts of the protozoan parasite *Abelspora portucalensis* Azevedo, 1987 (Microspora, Abelsporidae). Cysts were excised from parasitized crabs and sliced into small fragments which were fixed in 3% glutaraldehyde in 0.2 M sodium cacodylate (pH 7.2) at 4°C for 3 h, rinsed for about 2 h in the same buffer, and postfixed with buffered 2% OsO_4, at 4°C for 2 h. After dehydration in ethanol, the pieces were placed in propylene oxide and then embedded in Epon. Ultrathin sections were doubly stained with aqueous uranyl acetate and lead citrate (Reynolds) and examined under a JEOL 100 CXII TEM at 60 kV.

Morphometric analysis

Three animals were used for the quantitative analysis. Several cysts were extracted from each specimen and treated as described above, but only five blocks per specimen, chosen at random, were used. Only one grid was analyzed per block. Two negatives from the schizont, sporont, and sporoblast stages were taken per grid at magnifications of 16,000 or 20,000. Thus, a total of thirty cells of each stage was analyzed. The cell profiles were chosen at random as well. Prints were obtained and their individual final magnification was determined for calculation purposes.

A multipurpose test system, widely known as a standard morphometric technique (Weibel and Bolender, '73), with 84 lines, each 1.35 cm long, enabled the determination of the following parameters:

1. Volume density or fractional volume (V_v-%) of intracellular structures (excluding the rough endoplasmic reticulum [RER]), by using the formula (Weibel and Bolender, '73)

$$V_v = (Pi \times 100)/Pt$$

in which Pi is the number of points within the component profiles and Pt is the total number of test points laying within the cell profile.

2. Surface-to-volume ratio in connection with either the soma (S/V) or the nucleus (S/Vn) as well as the surface density of the nucleus in relation to the soma (Svn), using the formula (Weibel and Bolender, '73)

$$S/V, S/Vn, Svn = (4 \times i)/(Z \times Pi)$$

where i is the number of intersections with the soma (for S/V) or with the nucleus (for S/Vn and Svn) and Pi is the number of test points which lay over the soma (for S/V and Svn) or over the nucleus (for S/Vn). Z is the length of a test line according to magnification.

The nuclear absolute volume (Vn) was obtained with the formula (Palkovitz and Fischer, '68)

$$Vn = (\pi/6) \times L \times B^2$$

in which L is the major diameter of the nucleus and B is the length of the perpendicular that crosses L through its middle point, measured within the organelle outline. Out of the initial thirty negatives per stage, about ten were eliminated, because they were unsuitable for measuring the parameter L. The discarded negatives were replaced with the same number of other negatives to reach the former total. To calculate the somatic volume (Vs), the absolute volume of the nucleus was multiplied by 100 and the product was divided by the volume density of the nucleus. The multiplication of the mean value of Vs by the individual volume density of the cytoplasm and vesicles gives their absolute volume.

A grid consisting of 17 parallel lines spaced 1 cm apart was used to calculate the membrane surface concentration (MSC), or surface density of rough endoplasmic reticulum (RER) (i.e., the average number of square micrometers of RER per cubic micrometer of cytoplasm) by using the formula (Loud, '62)

$$MSC = (C \times M)/(L \times 1000)$$

in which C is the number of crossings between the profiles of RER and the grid lines, M is the individual print magnification, and L is the total line length that lies over the total cytoplasm. Finally, the mean surface of RER per cell (RER-S) was calculated by multiplying the MSC by the mean total volume of the cytoplasm.

To overcome the anisotropry, especially that patent on the sporont stage, in all tests each print was analyzed four times under each grid, with a rotation of 45° upon each reading. The mean value for each cell was used in the statistics.

Statistical analysis

The data were studied with standard statistical tests and presented as mean ± SE (standard error). For percentage values, the statistical significance of pair comparisons was carried out by analyzing the standard error of the difference; the critical ratio was then examined by a two-tailed test with a Z probability table. For absolute values, the significance of differences between means was analyzed by making use of the Students's t-Test (two-tailed test based on the t-Distribution). For both tests, the results under comparison were considered not significant for $P > 0.05$.

RESULTS

Qualitative findings

The different life cycle stages are found dispersed in the host cell cytoplasm. By division and differentiation the schizonts originate sporonts; these divide directly to form sporoblasts and, finally, these differentiate directly to spores.

The schizonts (Fig. 1) are uninucleated cells, pleomorphic (ranging from round to ellipsoid), sometimes with an irregular outline. The nucleus, showing a nucleolus, is usually ellipsoid, with a rather smooth contour, lacking conspicuous indentations. The cytoplasm (Figs. 2–5) contains numerous free ribosomes, cisternae of RER in which the ribosomes are loosely spaced, some vesicles with a lucent matrix, small areas with closely spaced opaque vesicles (Figs. 2, 3), which sometimes fuse to form short tubular-like structures, and finally some microtubules (Figs. 4, 5).

The sporonts (Fig. 6) are uninucleated round to ellipsoid cells, distinguished from the previous stage because the plasmalemma has an incomplete external amorphous electron-dense coat with associated vesicles in some regions. The nucleus has a nucleolus and follows the shape of the cell. A microtubular manchette differentiates in the nucleoplasm just prior to the division of the cell. The cytoplasm has the same organelles as the schizont, but the RER content seems to vary in a greater extent from cell to cell; moreover, it contains fewer opaque vesicles and microtubules.

The sporoblasts (Fig. 7) are elongated cells with an ellipsoid nucleus, somewhat regular in contour and presenting a nucleolus. The cytoplasm has the same organelles as noted for the previous stages, including some fortuitous microtubules, but the dense vesicles are rare and more dispersed.

The spores (Fig. 8) present the usual structures found in other microsporidian species (i.e., ellipsoid body surrounded with a thick wall, cytoplasm bearing a central nucleus, densely spaced free ribosomes and polyribosomes as well as the extrusion apparatus composed of the anchoring disc, polar filament, polaroplast and posterior vacuole).

Quantitative findings

The morphometric data are summarized in Tables 1–4.

Cellular volume and S/V (Tables 1, 3)

The cell volume increases from the schizont to the sporont stage ($P < 0.02$); it decreases markedly if the sporoblast is compared with previous stages ($P < 0.001$). The S/V remains stable in the two first stages and increases significantly ($P < 0.001$) in the last one.

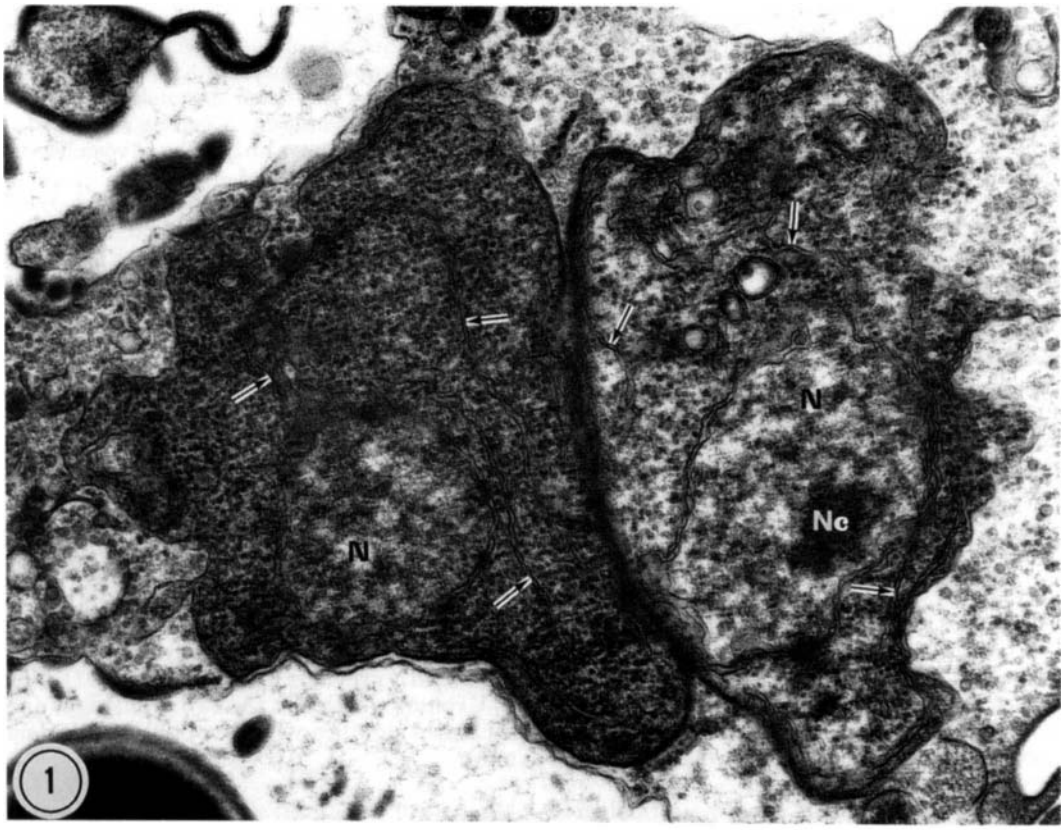
Nucleus (Tables 1–3)

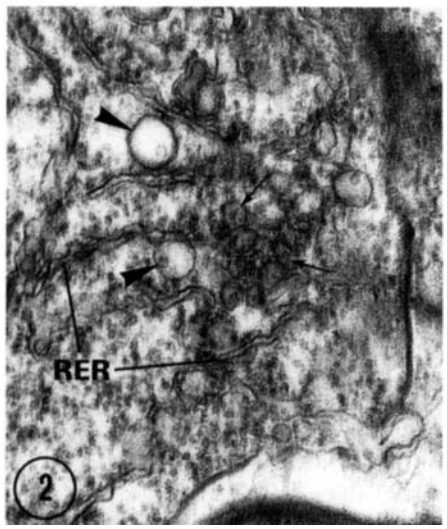
Concerning the absolute volume of the nucleus, the first two stages show no statistically significant difference; the opposite is true if either is compared with the sporoblast ($P < 0.001$): the latter then exhibits less volume.

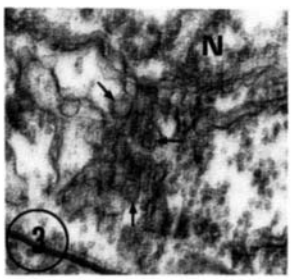
During sporogenesis, the organelle diminishes its proportional volume. Sporont and sporoblast do not differ significantly from each other; nevertheless, either one differs significantly from the schizont ($P < 0.001$), the latter stage being larger. Additionally, the Svn maintains its magnitude; however, the S/Vn increases and significant differences are seen between schizont/sporont ($P < 0.01$), schizont/sporoblast ($P < 0.001$) and sporont/sporoblast ($P < 0.01$).

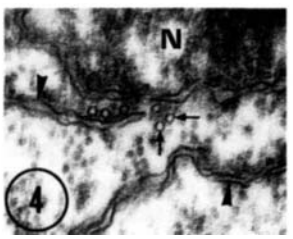
Nucleolus (Tables 1, 2)

During sporogenesis, the absolute volume of the nucleolus decreases during the transition from sporont to sporoblast ($P < 0.01$). The schizont and sporont stages do not differ significantly; however, the schizont is quite distinct ($P < 0.001$) from the sporoblast stage. The relative volume also decreases









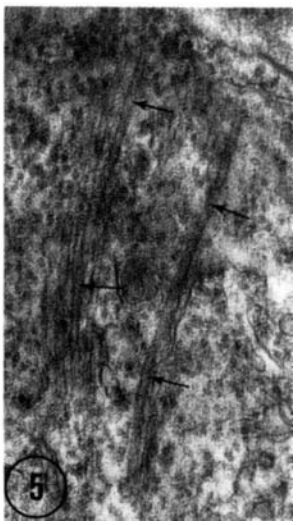


Fig. 1. *Abelspora portucalensis*. Section of two schizonts lying side by side. Note the ellipsoid nucleus (N) with a prominent nucleolus (Nc) (right), and also the loose array of the rough endoplasmic reticulum (arrows). ×39,000.

Fig. 2. *Abelspora portucalensis*. Detail of the schizont cytoplasm, displaying the rough endoplasmic reticulum cisternae (RER), vesicles with clear matrix (arrowheads), and vesicles with a dense core (arrows). ×48,000

Fig. 3. *Abelspora portucalensis*. Schizont: detail of a group of opaque vesicles (arrows). N, nucleus. ×48,000.

Fig. 4. *Abelspora portucalensis*. Schizont: detail of cross-sectioned microtubules (arrows) in close association with the nucleus (N) and rough endoplasmic reticulum (arrowheads). ×48,000.

Fig. 5. *Abelspora portucalensis*. Longitudinally sectioned microtubules (arrows) in the schizont cytoplasm. ×48,000.

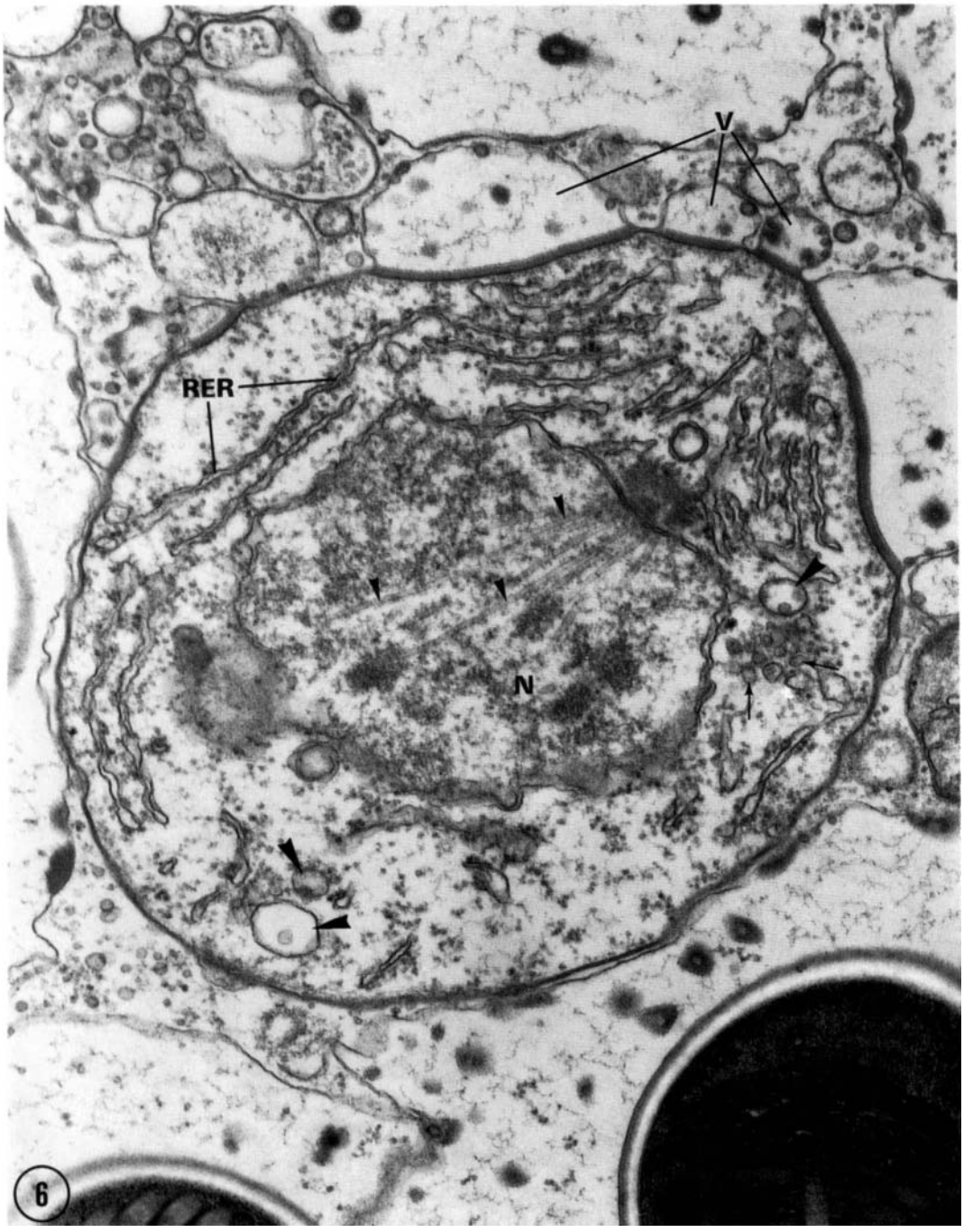


Fig. 6. *Abelspora portucalensis*. Section of a sporont. In the nucleus (N) a microtubular manchette (short arrowheads) can be detected. In the cytoplasm note the presence of clear (long arrowheads) and opaque (arrows) vesicles, the stacking of the reticulum (RER), and also the vesicles (V) associated with the external coat. ×43,200.

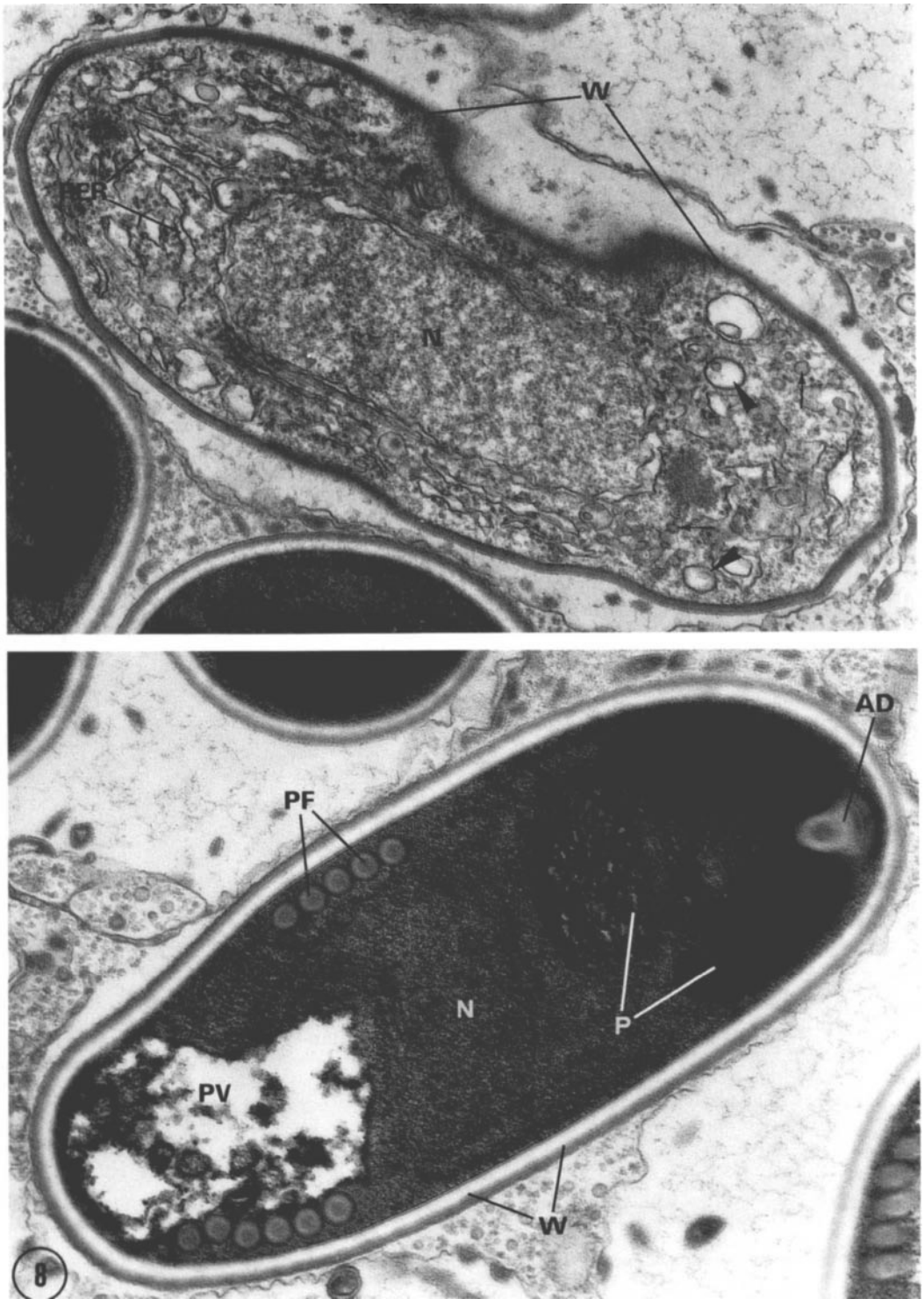


Fig. 7. *Abelspora portucalensis*. Section of a sporoblast showing an ellipsoid nucleus (N) and cytoplasm surrounded with a wall (W) of dense material. Clear vesicles (arrowheads), opaque vesicles (arrows), and reticulum (RER) are seen dispersed in the cytoplasm. ×40,000.

Fig. 8. *Abelspora portucalensis*. Section of a mature spore displaying the anchoring disc (AD), the polaroplast (P), and the polar filament (PF) helically coiled around the posterior vacuole (PV). The nucleus (N) is barely perceptible. W, wall. ×40,000.

TABLE 1. Absolute volumes (μm^3) of cells and organelles of the different stages of Abelspora portucalensis

Stage	Schizont	Sporont	Sporoblast
Cell	5.04 ± 0.18	5.92 ± 0.30	2.62 ± 0.27
Nucleus	1.66 ± 0.11	1.61 ± 0.13	0.63 ± 0.10
Nucleolus	0.06 ± 0.01	0.04 ± 0.01	0.01 ± 0.004
Cytoplasm[1]	3.35 ± 0.05	4.25 ± 0.06	1.97 ± 0.04
Vesicles	0.03 ± 0.01	0.06 ± 0.01	0.02 ± 0.01

[1]Excluding the vesicles with a clear matrix.

TABLE 2. Volume densities (V_v-%) of organelles of the different stages of Abelspora portucalensis

Stage	Schizont	Sporont	Sporoblast
Nucleus	32.94 ± 0.99	27.20 ± 1.07	24.06 ± 1.33
Nucleolus[1]	3.60 ± 0.69	2.22 ± 0.68	0.81 ± 0.57
Cytoplasm[2]	66.52 ± 1.00	71.79 ± 1.08	75.11 ± 1.35
Vesicles	0.54 ± 0.16	1.01 ± 0.24	0.83 ± 0.28

[1]In relation to the nucleus.
[2]Excluding the vesicles with a clear matrix.

gradually; the only significant difference is that perceived between the schizont and sporoblast stages ($P < 0.01$).

Cytoplasm (excluding the vesicles with a clear matrix; Tables 1, 2)

The changes of the absolute cytoplasmic volume follow the same pattern as those of the cell volume, with highly significant differences ($P < 0.001$) between any pair of comparisons concerning the three stages. The relative volume increases during sporogenesis, with highly significant differences ($P < 0.001$) between the schizont and any of the other two stages, although these do not differ significantly among themselves.

Vesicles (with clear matrix; Tables 1, 2)

The absolute volume of vesicles rises significantly from the schizont to the sporont stage ($P < 0.05$) and then decreases markedly from the sporont to the sporoblast stage ($P < 0.01$) to reach a value within the range of that of the schizont. Despite the apparent differences between the mean values, the relative volume does not change significantly in the three stages.

MSC (Table 4)

The sporont stage attains the highest value of MSC; however, it differs significantly only from the schizont stage ($P < 0.05$). No significant difference occurs between the schizont and sporoblast stages.

TABLE 3. Surface-to-volume ratios ($\mu m^2/\mu m^3$) of cell and nucleus as well as surface density of the nucleus ($\mu m^2/\mu m^3$) of the different stages of Abelspora portucalensis

Stage	Schizont	Sporont	Sporoblast
S/V	2.40 ± 0.12	2.17 ± 0.08	3.24 ± 0.12
S/Vn	3.89 ± 0.16	4.60 ± 0.20	5.76 ± 0.37
Svn	1.26 ± 0.06	1.24 ± 0.06	1.24 ± 0.14

TABLE 4. RER parameters of the different stages of Abelspora portucalensis: *MSC ($\mu m^2/\mu m^3$) and RER-S (μm^2)*

Stage	Schizont	Sporont	Sporoblast
MSC	5.01 ± 0.28	6.07 ± 0.38	5.56 ± 0.49
RER-S	16.93 ± 0.93	26.16 ± 1.64	11.06 ± 0.98

RER-S (Table 4)

The RER-S changes significantly during sporogenesis, with all the three stages differing in pair comparisons ($P < 0.001$). The sporont stage presents the highest value.

DISCUSSION

The Golgi apparatus of the microsporidia is thought to be dispersed in the cytoplasm as groups of small opaque vesicles, each one limited with a single membrane (Vávra, '65, '68, '76). Some authors recognize, however, a Golgi composed of cisternae, vesicles, and vacuoles (Morrison and Sprague, '81; Sprague and Vernick, '74; Vernick et al., '77). Some confusion in identifying the Golgi and RER may arise (Vávra, '76) because of the failure of some fixation procedures to reveal the association among membranes and ribosomes. Our qualitative data of *Abelspora portucalensis* reveal groups of vesicles in the cytoplasm, the features of which are similar to those above mentioned; being so, they may represent the Golgi. This organelle, not previously studied by Azevedo ('87), is thought to be involved in the formation of the extrusion apparatus—for example, by coalescence of vesicles (Jensen and Wellings, '72; Loubés and Maurand, '76; Toguebaye and Bouix, '83; Vinckier, '75). The dispersion and scarcity of this component, noted in the sporoblast stage of *A. portucalensis*, may be the result of movement and fusion of the vesicles to form the referred apparatus.

We here report the occurrence of microtubules in the cytoplasm of all stages, except spores. These organelles are rarely observed in the microsporidia (Vávra, '76) and, perhaps due to their depolymerization during

fixation, they have not previously been reported in the present species (Azevedo, '87).

According to Vávra ('76), both conventional types of endoplasmic reticulum (rough [RER] and smooth [SER]) occur in microsporidian cells. In the several developmental stages of the present species, the RER is practically all in the form of cisternae, with occasional vesicles seen throughout the cytoplasm. In the sporont and sporoblast stages the RER cisternae are arranged in stacks, parallel to the cell and nuclear surfaces, whereas in the schizont stage they are dispersed, not stacked; the latter situation is in accordance with previous observations on other species (Cali, '71; Lom and Corliss, '67; Youssef and Hammond, '71). As to the quantitative changes, Vávra ('76) noted that there are more cisternae in sporulation stages; for example, studies by Toguebaye and Marchand ('84) seem to reveal that in *Nosema henosepilachnae* the sporont has a greater number of RER cisternae than does the schizont. Based on our qualitative approach, it is difficult to affirm with certainty that one stage differs from the other (especially when comparing the schizont with the sporont), because of differences in cell size and array of the cisternae. The studies made on *Abelspora portucalensis* (Azevedo, '87) seem to suggest that in the sporoblast the RER is less evident. Our quantitative results agree only in part with previous findings; the sporont stage is undoubtedly the one presenting the greatest amount of RER (although not significantly differing from the sporoblast in what concerns the MSC); the schizont and sporoblast are alike in this matter since they have the same "concentration" ($\mu m^2/\mu m^3$) of RER. The sporoblast has, however, a lesser total surface of RER whenever compared with either the schizont or the sporont; nevertheless, this fact is due to its inferior total volume.

In microsporidia, the SER is possibly represented by vesicles with a transparent content which are freely distributed in the cytosol (Vávra, '76). According to Vivier and Schrevel ('73), the vesicles of the SER are more abundant and larger in those cells in which the nucleus is undergoing karyokinesis. Our data show a population of larger vesicles with a clear matrix; there are significant differences in their absolute volume, but not in the relative one. If this kind of vesicle really represents the SER, then, based on the latter finding, we are tempted to suggest that metabolic demandings on the organelle remain relatively constant throughout sporogenesis.

It is interesting to note that although the absolute volume of the sporont cell is greater than that of the schizont, the ratio S/V keeps constant. Does this fact indicate that the parameter has to hold at an optimum level so that the parasite cell establishes its metabolic "dialogue" with the host cell? The soporoblasts, in which contact with the immediate environment (host cell cytoplasm) is slowly prevented by the formation of the spore wall, increase the S/V. The latter fact can eventually support that hypothesis. The increment of the S/V in sporoblasts and, of course, spores (deduced by their shape) can also be correlated with the dispersion strategy of microsporidia; an ellipsoid shape could not only facilitate the transit through the alimentary tract of a possible new host, but also could offer a surface sufficient for recognizing the local environmental stimuli, namely those associated with spore germination.

Another finding worth mentioning deals with the Svn. This parameter does not significantly change, even though the absolute and relative volumes and the surface-to-volume ratio of the nucleus do. The immutability of the Svn, achieved by the development in opposite ways of the V_v (decrease) and S/V (increase) of the nucleus, may again reflect a vital need—that is, the maintenance of a fixed surface of the nucleus per volumetric unit of cytoplasm. This could be important, for example, in effecting the movements of mRNA and/or enzymes to and from the nucleus. Still in relation to the nucleus, the nucleolus diminishes its relative and absolute volumes during sporogenesis. This fact can be explained, at least in part, if we take into account that the cytoplasm of the different stages, as generally happens in microsporidia (Vávra, '76), becomes congested with ribosomes; therefore, it is logical to note that their rate of production slowly diminishes with a concomitant regression of the nucleolus.

In conclusion, the stereological approach seems to be useful in characterizing particular aspects of cell differentiation leading towards the production of spores of *Abelspora portucalensis*, and eventually other microsporidia. In the future, similar methodology can also be applied for quantifying morphological alterations in toxicologic studies on microsporidia; such alterations were re-

ferred, for example, on *Glugea anomala* by Schmahl and Mehlhorn ('89) based solely in qualitative data.

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