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Effects of Fixation, Dehydration and Staining on Dimensions of Myxosporidan and Microsporidan Spores

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Abstract

The effects of fixation, dehydration and staining on the morphological dimensions of myxo- and microsporidan spores were tested. Seven fixatives, two dehydrants and five stains were tested. Ten % formalin produced the least shrinkage and provided the best cytological detail of fixed material in both types of spores. All fixatives caused shrinkage of myxosporidan spore length and polar capsule length. Spore capsule width and polar capsule width were unaffected by 10% formalin. Ethyl alcohol caused no significant change in spore width. Microsporidan spore length shrunk with all fixatives, but spore width was generally unaffected. Dehydration, with either isopropyl alcohol or acetone, produced additional, significant shrinkage. The influence of stains on spore size was negligible. Heidenhains iron hematoxylin followed by eosin, and Mallory's analine-blue collagen stain, effectively stained myxo- and microsporidan spores.

Introduction

In both the Myxosporida and Microsporida, spore dimensions and spore morphology serve as a major basis for distinguishing two species. Variation of spore size due to age (or life cycle), sex, organ, or species of host are not clearly understood. This greatly complicates the problem of using spore dimensions as a taxonomic aid. Lack of knowledge of the effect of fixation and staining on spore dimensions has complicated this problem. Blunck[2] said microsporidan spore size varies in different stages of the host and from different tissues in the same host. Thomson[18] found no significant difference in mean spore size from a single host, even from widely separated areas. He and Walters[19] stated that the length of spores varied with the age of the host. However, Summerfelt and Warner[16] found that age of host had no effect on spore dimensions. Kudo[9] cautioned investigators to report the condition that spores were subjected to before and during measurement. Thomson[18] said that all spores should be measured in distilled water to eliminate artifacts caused by fixation and staining. It should be noted here that

[1] Contributed material on Myxosporida: current address, Lincoln Land Community College, Springfield, Illinois.

[2] Contributed material on Microsporida.

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extended periods of time in distilled water may cause swelling because the spores are hypertonic to the water. As he investigated this problem in the Myxosporida, Kudo[8] subjected spores of *Leptotheca ohlmacheri* Ohlmacher and Whinery, 1893, to seven fixatives. From this study he determined that "ordinary fixation" decreased sutural diameter and breadth of spores approximately 14-22%, caused the entire spore body to shrink, rendered the polar filament invisibile, and varied in the amount of shrinkage produced depending upon the fixative used.

Of the several fixatives tested, Kudo noted that 50% alcohol produced the least shrinkage and 4% formalin caused the greatest overall shrinkage. Kudo did not determine whether a portion of the shrinkage he attributed to fixation was due to staining, dehydration, or to the combined influences of staining and dehydration.

Influence of fixatives on spore size have been noted in the Myxosporida (Bond[3]; Fish[4]; Iverson[7]; Wyatt and Pratt[20]; Lewis and Summerfelt[11]; Hoffman, Putz and Dunbar[5]) and the Microsporida (Walters[19]; Thomson[18]; Summerfelt[15]; and Summerfelt and Warner[16]). With the exception of the study by Hoffman, Putz and Dunbar, these workers reported that fixation affects shrinkage in Cnidosporidan spores. In their study, *Myxosoma cartilaginis* showed an increase in valvular length and width following fixation.

Meglitsch[13] compared shrinkage of formalin (4%) fixed spores of *Myxidium kudoi* in distilled water, cedar wood oil, and Canada balsam. He concluded that there was greater shrinkage of the spore in transfer from distilled water to balsam than was produced by fixation. It is not known whether the differences noted by him were due in part to dehydration in balsam and cedar wood oil as compared to a lack of dehydration in distilled water. Also, a difference in the refractive indices of each media may influence variations in the measurements obtained.

Ross[14] found the difference of refractive indices between saline and Canada balsam caused measurements of cells to vary by less than 0.75%. Since the difference in refractive indices between the saline and the mounting medium (Permount) used in the present study was less than those used by Ross, it can be assumed that the refractive differences had no significant effect on the measurements.

Lillie[12] reported formaldehyde fixation may not harden certain cytoplasmic structures sufficiently for paraffin embedding. Similarly, Humason[6] found formaldehyde may incompletely preserve cells. She noted that shrinkage may take place if dehydration, clearing and infiltration are conducted too soon after fixation. Baker[1] observed that formaldehyde swelled gelatin/albumin to 123% of the original volume, but whole liver retained 99% of its original volume. When the liver was dehydrated and embedded in paraffin, shrinkage had reduced its initial volume by 32%.

Baker[1] noted that ethanol (96%) shrinks gelatin/albumin more than any fixative except acetone. Mercuric chloride (saturated aqueous) reduced the volume of gelatin/albumin and whole liver less than 10% and potassium dichromate (3% aqueous) increased gelatin/albumin to 160% of the original volume while whole livers remained unchanged. Acetic acid (5% v/v) swelled gelatin/albumin more than any other fixative. He observed that regardless of the fixative (ethanol, mercuric chloride, potassium dichromate, or acetic acid) dehydration resulted in shrinkage. Thompson[17] noted that, in general, the anhydrous fixatives, such as acetone and absolute alcohol, can be expected to cause considerable shrinkage. Zenker's and Bouin's fluids caused little shrinkage or swelling and acetic acid fixatives produced the most marked increase in volume.

The objectives of this study were to study the comparative effects of fixation, dehydration and staining on Myxo- and Microsporidan spores.

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Materials and Methods

Mature spores of *Plistophora ovariae* Summerfelt, a microsporidan from *Notemigonus crysoleucas* and *Myxosoma* species, an undescribed myxosporidan from *Gambusia affinis* were subjected to seven different fixatives: absolute ethyl alcohol, Bouin's 10% formalin, Gilson's, Gomori's, Kaformacet (12.5 ml acetic acid, 25 ml formaldehyde, 6.5 g $K_2Cr_2O_7$, 212.5 ml H_2O) Schaudinn's and Zenker's; and, to five separate stains: Acetocarmine, Geimsa's, Heidenhain's iron hematoxylin and eosin, Mallory's aniline-blue, and a metachromatic blue (1% methylene blue, 1% toluidine blue and 0.5% sodium borate). Spores were retained in the fixatives for a period of twenty-four hours at 7 to 9° C.

In addition to measurement of spores in the individual fixatives, mean dimensions of the main spore characteristics were determined for spores subjected to all of the possible combinations of fixative and stain pairs. The influence of dehydration was investigated independent of the stains. Samples of fixed spores were passed through an alcohol series (35%, 50%, 70%, 95%, and 100%), transferred to toluene and mounted in permount. Dehydration with acetone consisted of 3 changes of acetone, followed by transference to toluene and mounting media. The influence of hydration and dehydration on spore dimensions was evaluated by transferring the spores through descending and/or ascending alcohol series.

Fresh spores were obtained by rupturing a sample of infected host tissue in Locke's solution. A set of measurements of an individual spore characteristic was based on a sample of twenty spores. Each set of measurements was replicated on a second sample of spores. The spores of *P. ovariae* and *Myxosoma* sp. were measured after the methods of Kudo.[8,10] Duncan's multiple range test was employed in the analysis of variance. A 0.05 level of significance was accepted as denoting significant differences. A comparison of the pooled means of replicates was made to determine if: (1) the dimensions of fresh spores differed significantly from fixed spores, from fixed and dehydrated spores, and from fixed, dehydrated and stained spores; (2) the dimensions of spores varied with the fixative used; (3) the dimensions of spores differed significantly as a result of the dehydrating chemicals (isopropanol and acetone); (4) and the dimensions of spores varied with the kind of stain.

Results

Myxosporidan Spores

Spore length: Each of the seven fixatives that were tested produced some degree of shrinkage in the length of the spores. Ten percent formalin resulted in the least shrinkage while Bouin's, Kaformacet, ethyl alcohol, Zenker's and Gomori's fluids caused an intermediate amount of shrinkage. The greatest amount of shrinkage was produced by Gilson's and Schaudinn's fluids (Figure 1). Both Gilson's and Schaudinn's contained acetic acid, an agent more frequently associated with swelling than with shrinkage. It would appear that the relative volume of acetic acid was insufficient to prevent shrinkage in the spores. Means of spore length of all fixatives were significantly smaller than the lengths of fresh spores.

The overall mean length of fixed spores was significantly larger than the mean length of the fixed and dehydrated spores (Figure 2). The mean length of dehydrated spores and dehydrated and stained spores was approximately the same (Figure 2). No significant difference in shrinkage ocurred among stains.

Spore width: Formaline and ethyl alcohol produced no significant effects on the width of the spores. All other fixatives caused shrinkage sufficient to produce a statistically significant difference from the mean width of fresh spores (Figure 1). Shrinkage in the width of spores was most pronounced in Bouin's fluid. The striking reduction in spore width accompanying the carmine stain is not believed to be due to the stain but to natural variation in spore sizes (Figure 2).

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Polar capsule length: Length of the polar capsule was least altered by formalin and Kaformacet fixatives. The greatest shrinkage of this structure occurred in Bouin's and Gilson's fluids. The remaining fixatives produced approximately the same shrinkage of the polar capsule. All of the fixatives caused sufficient shrinkage to differ significantly from the mean length of the polar capsule of fresh spores (Figure 1).

Polar capsule width: With the exception of ethyl alcohol, the reduction in the width of the polar capsule by the fixatives

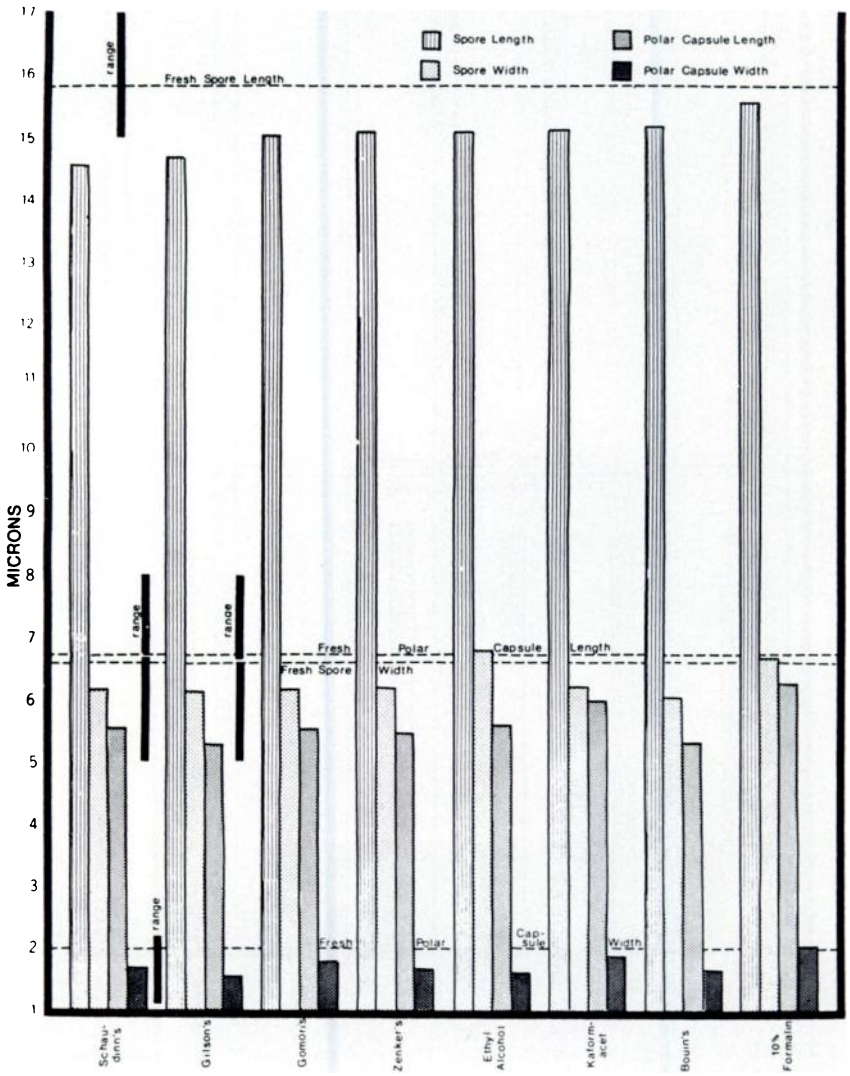

FIGURE 1. *Changes in dimensions of Myosporidan spores and polar capsules caused by fixation.*

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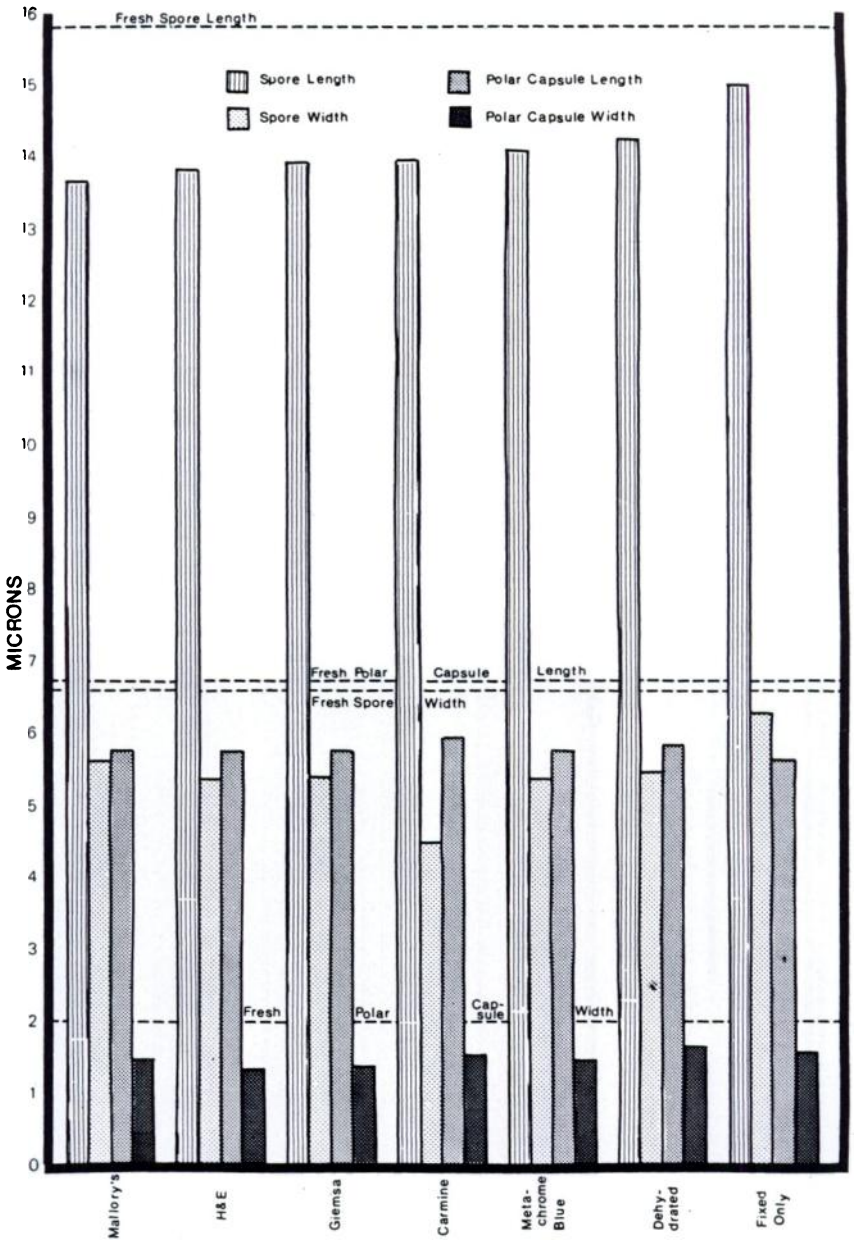

FIGURE 2. *Changes in dimensions of Myxosporidan spores and polar capsules caused by fixation, dehydration, and staining. Each column represents samples from all fixatives.*

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closely paralleled the pattern of shrinkage in the length of the polar capsule (Figure 1). Width and length of the polar capsule also responded similarly to the stains and to dehydration. No significant variations in the mean widths or lengths of polar capsules were associated with specific stains (Figure 2). Although nonsignificant statistically, the mean width and length of the polar capsules after fixation and dehydration, were slightly greater than comparable means obtained after fixation only (Figure 2).

Most satisfactory fixation of Myxosporidan spores was obtained with 10% formalin. Wet mounts of formalin-fixed spores were particularly useful when examining the polar capsules and sporoplasm nuclei. Fixation in formalin for a period of twenty-four hours was not sufficient to prevent shrinkage of the cytoplasm in the process of dehydration. Before initiating dehydration, a longer period of fixation is recommended. Kaformacet and Gomori's, although accompanied initially by more shrinkage than formalin, did not permit the cytoplasm of spores to be noticeably shrunken by dehydration.

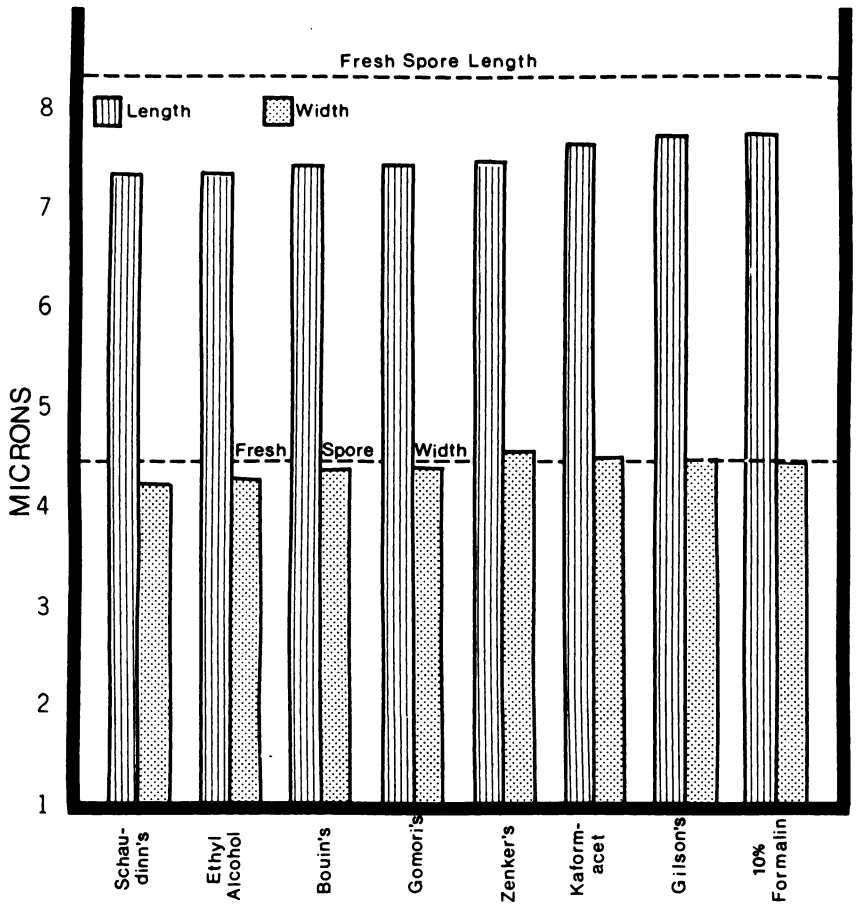

FIGURE 3. *Changes in dimensions of Microsporidan spores caused by fixation.*

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Differentiation of myxosporidan spores was accomplished most effectively through the use of Heidenhain's iron hematozylin and eosin counterstain, and by Mallory's analine-blue collagen stains. Neither stain was of value in rendering the polar filaments visible.

From pooled data on spore dimensions from all seven fixatives, the average reduction in spore length and width was 5% for both the mean length and width of fresh spores. An additional loss of 5% and 13% in the length and width of the spore, respectively, was attributed to the influence of dehydration. Under the influence of fixation, the polar capsules showed a decrease in length of 16% and a loss of 15% in width. Dehydration resulted in no additional loss in polar capsule length, but the width of the polar capsule shrunk an additional 6%.

Microsporidan Spores

Spore length: Shrinkage occurred in all instances of fixation but was less pronounced in 10% formalin, Gilson's and in Kaformacet than in the other fixatives (Figure 3). The mean length of spores

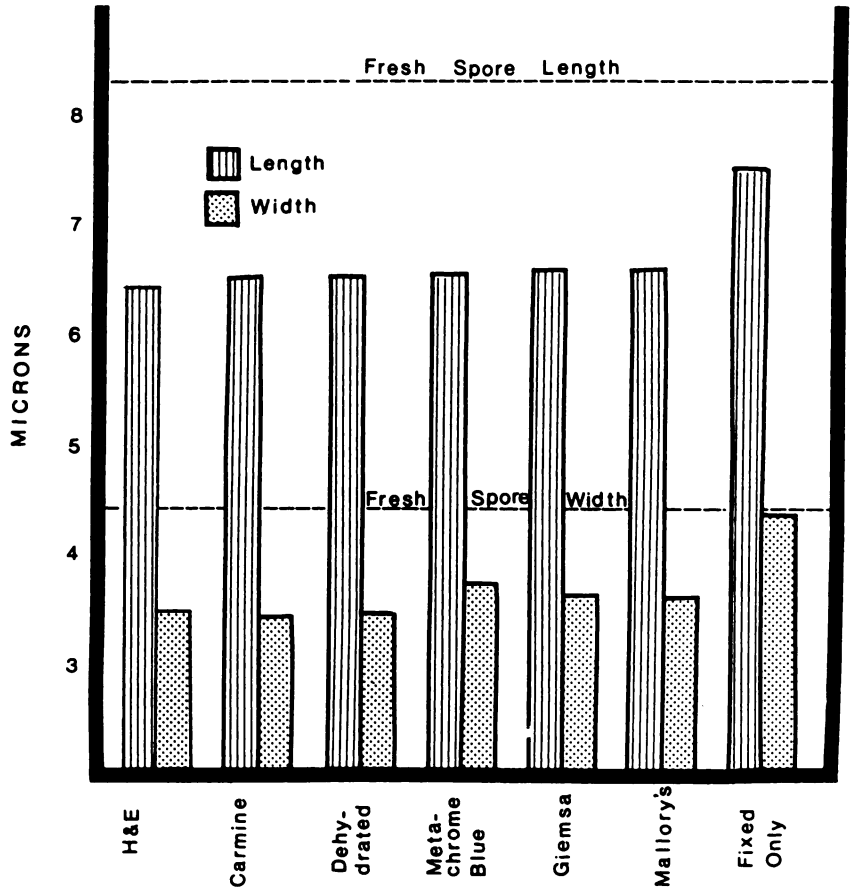

FIGURE 4. *Changes in dimensions of Microsporidan spores caused by fixation, dehydration and staining. Each column represents samples from all fixatives.*

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in each of the fixatives differed significantly from the mean length of fresh spores. When the shrinkage in the lengths of the spores in the various fixatives was ranked from the greatest mean shrinkage to the least mean shrinkage, it was found that 10% formalin (least), Kaformacet (moderate), and Schaudinn's (greatest) fluids ranked the same in their influences on the Micro- and myxosporidan spores (Figures 1 and 3).

Spore width: Of the various fixatives, only Schaudinn's and ethyl alcohol produced significant shrinkage in the width of the spore (Figure 3). As to the width of stained spores, three homogenous sets were observed: Mallory's and Giemsa's; H. & E., Carmine and dehydrated but no stain; and Metachromatic blue (Figure 4). Each of these sets differs significantly from the other two sets. The total range of the means of widths of the spores for these sets, however, is less than 0.5 micron.

Formaldehyde, as with the myxosporidans, proved to be the best fixative on the basis of the ability to resolve various spore structures after fixation. The internal morphology was least distinguishable in spores following fixation with 100% ethyl alcohol. Spores fixed in Gilson's and in ethyl alcohol showed the clearest internal structures following fixation and dehydration.

Mallory's analine blue and Heidenhain's H. & E. both stained the spores well and provided good pictures of their internal morphology. The sharpest stain was obtained with Gilson's fixative and Mallory's stain. Acetocarmine and Giemsa's did not provide good differentiation of spore morphology. The metachromatic blue stained the spores well and made them easy to distinguish even at lower magnifications. However, spores stained with metachromatic blue caused more eye fatigue than the other stains.

Conclusions

1. Dimensions of fresh myxosporidan and microsporidan spores were decreased by fixation.

2. Dehydration of fixed spores contributes additional shrinkage that may be equal to or greater than the shrinkage caused by fixation.

3. Neither fixation or dehydration reduces the different dimensions of the spores by equal proportions.

4. Shrinkage caused by dehydration with alcohol or acetone was essentially the same.

5. The influence of staining on the dimensions of myxo- and microsporidan spores is negligible.

6. Internal structure may appear different following fixation with different fixatives.

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