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Notes on the Effects of Pretreatment of Spores on the Iodinophile Vacuole of *Henneguya exilis*[1]

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Abstract. Spores of *Henneguya exilis* from the gills of *Ictalurus punctatus* were exposed to Lugol's solution, Best's carmine, Bauer-Feulgen reaction, and Mitchell's ammoniacal silver nitrate techniques in smears and sections. All of the techniques recognized as specific for glycogen stained the iodinophile vacuole selectively but not specifically, but adjustments to techniques were required. Fresh spores held at room temperatures or chilled for 24 hours were similar insofar as the iodinophile vacuole is concerned, and freezing had little effect. A week or more at room temperature after chilling or freezing for one day resulted in more diffuse staining reactions.

A characteristic feature of the Myxobolidae (Myxosporida) is the presence of an iodinophile vacuole in the sporoplasm. It is found only in the Myxobolidae, and is the only distinguishing feature between that family and Myxosomatidae. Its usefulness as a taxonomic character has been well-established. In some species, however, the iodinophile vacuole is reported to be very difficult to demonstrate. Thélohan (1895) found this in *Myxobolus dispar*; Gurley (1893) in *M. transovalis,* and Lewis (1968) in *M. argenteus,* for example. Diffuseness of the reaction of the vacuole with iodine, or indistinctness of outlines of the vacuole have been reported by Gurley (1893) for *M. globosus,* and have been seen sometimes in this laboratory. This study was undertaken to determine whether the appearance of the iodinophile vacuole is sensitive to temperatures and storage, and to attempt to develop more dependable techniques for its demonstration.

MATERIALS AND METHODS

Spores of *Henneguya exilis,* obtained from cysts in the gills of *Ictalurus punctatus* taken from the Des Moines River during the summer of 1968 near Knoxville, Iowa, were used for the study. Hosts were brought back to the laboratory alive, or if they died during the trip, were stored at 4° C in a refrigerator, or were deep-frozen.

Fresh spores were exposed directly to Lugol's solution, or infected tissues were fixed in alcohol-formalin (9 parts absolute alcohol to 1 part neutral formalin), picro-alcohol-formalin (9 parts absolute alcohol saturated with picric acid to 1 part of neutral

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formalin). The latter fixative was used with the ammoniacal silver technique, mentioned below.

Spore suspensions were examined with Lugol's solution at room temperature, and after storage at room temperature, chilling, or deep-freezing. Tissues for sectioning were fixed immediately after removal from freshly-killed hosts, or from hosts that had been chilled or deep-frozen. Suspensions were made of fixed spores, and smear preparations were stained in the same manner as sections.

Permanent mounts of smears or sections were made using Lugol's solution, Best's carmine, the Bauer-Feulgen reaction (Bensley, 1939), or an ammoniacal silver nitrate technique (Mitchell, 1944).

Effects of Pretreatment

A suspension of fresh spores, immediately after staining with Lugol's solution, gives an impression of well-stained iodinophile vacuoles, but when the preparation is examined closely, some of the spores are found to have well-stained vacuoles with sharp outlines, while the vaculor margins of others are somewhat indistinct, or the iodine has stained a region of the sporoplasm more or less diffusely. A few spores have no iodine-stained region in the sporoplasm (Table 1). Only 78.5% of the spores have sharply outlined vacuoles, while 20.6% have a diffuse reaction. Spores kept at room temperatures for 24 hours do not change significantly, and spores that are chilled for a day or a week are essentially comparable to fresh spores. Spores taken from hosts that have been frozen for a day show more diffuse reactions, and fewer vacuoles with sharp outlines.

Table I. Effect of Spore Pretreatment on the Appearance of the Iodinophile Vacuole.

Conditions	N	Percentages with Vacuoles Sharp	Indistinct Recation	No Reaction
fresh, stained immed.	290	78.5	20.6	0.7
room temp., 24 hrs.	221	82.0	18.1	
chilled, 24 hours	200	77.5	21.5	1.0
chilled, 1 week	200	78.0	22.0	
deep frozen, 24 hrs.	300	63.3	34.7	2.0

An increased number of diffuse reactions is seen when spore suspensions made from chilled or frozen hosts are kept at room temperature (Table II). There is some indication that spores from frozen hosts do not change as rapidly as those from chilled hosts, but this is equivocal as an unexpectedly high number of spores with sharply outlined vacuoles were seen in samples held for a week at room temperatures after initial freezing.

It seems evident that the spores of *Henneguya exilis* contain an

iodinophile vacuole that does not change greatly during the first 24 hours, whether kept at room temperature, chilled, or deep-frozen, although longer exposures to room temperature do result in a decline of sharply-outlined vacuoles. It seems safe to conclude that in species like this one, differences in the stainability of the iodinophile vacuole are not likely to be an artefact if unfixed material is used, nor is any probable pretreatment before fixation likely to affect results.

Table II. Effect of holding spores at room temperature after chilling or freezing on iodinophile vacuole appearance.

Pretreatment	Time at Room Temperature	N	Percentages with Vacuoles sharp	Indistinct recation	No reaction
Chilling	24 hrs.	130	79.1	12.3	8.5
	1 week	205	57.6	42.4	
Freezing	24 hrs.	246	52.7	47.2	
	1 week	200	77.5	22.5	
	2 weeks	200	56.3	43.7	

Changes in the appearance of spores have been followed by Auerbach (1910), Thélohan (1895), and Bond (1938), in water suspensions and in saline. Although a good deal of information is available, the modifications of the iodinophile vacuole have not been described in detail. The gradual increase in diffuse staining suggests that vacuolar contents may be absorbed gradually, perhaps as an energy source for slow spore metabolism. If this should prove to be the case, the condition of the iodinophile vacuole might prove to the useful in estimating spore viability. A great deal more work will be needed, however, before any such method can be validated.

Vacuolar Reactions to Glycogen Stains

Techniques used for ordinary haematoxylin staining of spores do not retain the vacuolar contents, and the iodinophile vacuole is an empty space. It is well known that fixatives are not equally suitable for stablizing various types of protoplasmic contents, and it is quite possible that some of the observations of iodinophile vacuoles that are difficult to demonstrate depend on the treatment of fixed material, and the type of fixative used.

As a rule, most investigators appear to have assumed that the contents of the iodinophile vacuole are glycogen, or some closely related substance. Lugol's solution has long been used as a "specific" stain for glycogen, though its specificity is by no means perfect, and comparison of material exposed to ptyalin digestion with un-

digested material is necessary if very definite conclusions are to be drawn. Fixed smears of spores, and sectioned material, were treated with Best's carmine, Bauer-Feulgen, and ammoniacal silver nitrate techniques to determine whether the iodinophile vacuole contained material that responded to other glycogen tests.

Adjustment of these techniques to use with myxosporidan spores proved difficult. The spore coat is resistant to the passage of many reagents, and reaction times adequate for tissue cells do not give good results with spores. None of these techniques have been adjusted perfectly to the staining of spore vacuoles. All of them do stain the iodinophile vacuole, but under conditions that reduce their specificity.

Doubling the time of staining with Best's carmine, from 20 minutes to 40 minutes, gives a satisfactory recation, but the spore coat and polar capsules are also stained. Lengthening the time of staining with the Bauer-Feulgen technique also demonstrates the iodinophile vacuole, but the spore coat and polar capsules are also stained. Exactly the same results are obtained with ammoniacal silver. Use of these techniques in combination with ptyalin digestion has not yet been undertaken, partly because we hoped to obtain a somewhat more specific recation first. While it is possible that some combination of time and strength might be achieved that would produce selective staining of the vacuole, the results obtained so far suggest that this is improbable. As conditions producing a reasonably good staining of the vacuole are approached, other parts of the spore also react.

Bond (1937) reported staining reactions of the spore coat, noting differences between immature and mature spores. With the standard Feulgen reaction, the mature spore coat was stained; Bond concluded that during the disintegration of the valvular nuclei, nucleic acids were probably deposited in the spore coat. Similar results have been obtained by Chakravarty, Maity, and Ray (1962, 1964). It is interesting that the spore coat reacts positively to the leucofuchsin, whether chromium trioxide or hydrochloric acid pretreatment is used.

All of the techniques that have been described as selective for glycogen react with the iodinophile vacuole, and it seems highly probable that the vacuolar contents will prove to be glycogen or some closely allied compound. Further work along this line is being conducted. In any case, it seems reasonable that when fixed material is to be used for staining with Lugol's for iodinophile vacuole demonstration, a fixative that will stabilize glycogen should be chosen. This might reduce the diffuse or equivocal reactions, and make assignment to the Myxobolidae or the Myxosomatidae more precise.

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