CHAPTER-II

CYTOCHEMICAL INVESTIGATIONS ON THE SPORES OF

STYLOCEPHALUS CONOIDES DEVDHAR.
INTRODUCTION

Spores form an important stage in the life-cycle of gregarines since they are the sole means of transmitting the infection to new hosts. Once they are extruded from cysts they lie exposed to stresses of various environmental factors such as light, temperature, humidity and infective microbes; as such they have to develop many suitable characters of survival value to withstand these odds till they are ingested by their natural host individuals.

A cursory survey of the literature on the cytochemical studies on gregarines points out:

crystalligerus (Collins, 1972), Gregarina blaberao (Mercier, et al., 1973), Gregarina cuneata, Stenophora conjugata, Stylocephalus mesomorphi (Amoji, 1975) and Stenoductus penneri (Ramachandran, 1976);

b) Most of the available literature on gregarine spores is of morphological and taxonomical interest (Watson, 1916, Kamm, 1922; Ray and Chakravarty, 1933, Chakravarty, 1935; Misra, 1941; Ganapati and Narasimhamurti, 1955; Uttangi and Desai, 1961, 1962, Desai and Uttangi 1962a&b; Devdhar, 1962; Amoji and Rodgi, 1970, 1972 and 1973; Patil and Amoji, 1979);

c) There are some cytochemical investigations on spores of some sporozoans other than gregarines, Eimeria tenella (Gill and Ray, 1954; Nath and Dutta, 1962); E. brunetti and E. acervulina, (Pattillo and Becker, 1955); E. acervulina (Horton-Smith and Long, 1963); Eimeria acervulina, E. necatrix, E. meleaa and E. grimitis (Vetterling and Doran 1969); E. stiedae (Trandsen, 1970); Myxosporidaceae spp. (Podlipsaev 1972 and 1974; Podlipsaev and Schulman 1978; Donec et al. 1978); and Sarcocystis sp. (Fayer and Thompson, 1975); and

d) There are only three brief reports on cytochemical studies of spores of the gregarines Geneiorhynchus aeschiae.
Iji Hoplorhynchus oligoicanthus (Stein, 1961), Enterocystis ensis (Boyleva 1963) and Diplocystis spp. (Loubes and Bouix, 1970) to the best of knowledge of the present author. Therefore, in the present work an attempt is made to investigate the cytochemical organization of the spores of one gregarine in detail. The spores of Stylocephalus conoides are selected for the study as they are large and easy to handle.

**MATERIAL AND METHODS**

A number of host beetles *Opatrum* sp. collected from Dharwar were maintained in the laboratory for a day or two on dried leaves of tamarind. From time to time the fecal matter of the beetles was examined under a binocular microscope for cysts of gregarines. The cysts so obtained were isolated from the fecal matter, were briefly rinsed in 0.1% formaldehyde, and were thoroughly washed in distilled water. After this, they were kept on moist blotting papers in covered embryo cups for development at room temperature. In about 24 hours the cysts showed freshly formed zygotes or immature spores. These zygotes or immature spores are considered as zero hour old spores for calculation of age in hours.
These fresh and immature spores (zygotes) formed the first batch of spores for the cytochemical investigation. Later, six more batches of spores were obtained at 12 hours intervals after 12, 24, 36, 48, 60 and 72 hours of development.

The spores of the second batch were grey in colour; the spores of the third batch were greyish black, and those of the subsequent batches were black due to black pigment in the spore coat. The spores were utilized to study the cytochemical organization by investigating the cytochemical localization of proteins, carbohydrates and lipids.

The methods followed to determine the chemical nature of the zygotes and spores are given below.

A. Proteins:


Smears of zygotes as well as of spores fixed in Carnoy's fluid for 15 minutes were thoroughly washed in absolute alcohol. They were then stained for 15 minutes with staining solution containing 10 mg. of Bromphenol blue and 1.0 gm. of HgCl₂ in 10.0 ml of alcohol (95%).
This was followed by washing for 20 minutes in 0.5% acetic acid (3 changes); then washing in water and in phosphate buffer (pH 7.0) for 3 minutes. The preparations were quickly dehydrated in alcohol series, cleared in xylol and mounted in Canada balsam.

2. **Action of boiling H₂O, dil. acid and dil. alkali** (Oser, 1965):

   Samples of spores were separately boiled in H₂O, dil. HCl and dil. KOH for 10 minutes to find out whether the spore coat dissolves in any of these media.

3. **Xanthoproteic test** (Oser, 1965):

   1 ml. of conc. HNO₃ along with a few cysts containing spores was heated for 10 minutes to observe if a yellow colour develops. After cooling, dilute ammonium hydroxide was added to it in excess to find out whether the yellow colour if developed in traces would deepen into an orange colour.

4. **Ninhydrin-Schiff reaction** : (Yasuma and Itchikawa, 1953):

   Spores fixed in Carnoy's fluid and washed in alcohol grades were brought to water. They were then treated with 0.5% ninhydrin alcoholic solution overnight followed by
staining with Schiff's reagent. After the routine dehydration permanent preparations were made.

5. **Performic Acid-Schiff reaction** (Pearse, 1961):

Smears of spores fixed in Carnoy's fluid and brought to water through alcohol grades were treated for 10-20 minutes with Performic acid solution prepared by adding 4 ml. of 30% H₂O₂ and 0.5 ml of conc. H₂SO₄ to 40 ml. of formic acid 98%. After a brief washing in water the spores were stained with Schiff's solution, washed in water, dehydrated and mounted in ucamperal.

6. **Masson-Fontana method (Hexamine-Silver variant)** (Pearse 1961):

Spore smears fixed in Carnoy's fluid and brought to water were treated with Gram's iodine for 10 minutes. After a thorough washing in distilled water, the smears were left for 24 hours in Fontana silver solution prepared as follows: To 20 ml. of 10% AgNO₃ was added strong ammonia drop by drop, till the granular precipitate so formed was just dissolved. The solution was diluted by adding 20 ml. of distilled water and stored in a dark bottle. After briefly rinsing in distilled water, the smears were fixed for 2 minutes in 50% sodium thio-sulphate, were rapidly
dehydrated, cleared in xylene and mounted in Canada balsam.

7. **Conn and Stumpf method (1969):**

The dark spores were treated with a few drops of conc. HNO₃ followed by the addition of a drop of conc. KOH.

8. **Bleaching methods (Pearse, 1968):**

A smear preparation of spores was treated with 40% peracetic acid for about 10 minutes. Another preparation was treated with 10% H₂O₂ for about 2 hours owing to its slower reaction.

B. **Carbohydrates**

1. **Lugol's Iodine method (Gurr, 1956):** Zygotes (fresh) and spores (12, 24, 36 and 48 hours old) preparations fixed in absolute alcohol for 30 mins. were brought to 70% ethanol. Excess of ethanol was carefully and thoroughly blotted out and the smears were immediately transferred to xylene and then to absolute ethanol followed by staining with Lugol's iodine (Iodine 1 gm; Potassium iodide 2 gms; H₂O 100 ml.) for 10 minutes. Excess of stain was poured off, the smears were thoroughly blotted, cleared in xylene and mounted in balsam.
2. **Gram's iodine method** (Gurr, 1956): Gram's iodine was prepared as follows: 0.2 gm of potassium iodide was dissolved in 10 ml of distilled water; in this solution 0.1 gm of iodine was dissolved. This solution was further diluted by adding 20 ml of distilled water. Cysts containing spores were boiled in dil. KOH for 30 minutes washed in ethanol (90%), and were treated with Gram's iodine for 5 minutes. After a brief washing in water, the cysts were finally mounted in glycerol jelly.

3. **Best's Carmine method** (Pearse, 1968): Zygotes and spores fixed in Carnoy's fluid were brought to water and were stained with Best's Carmine solution for about 20 minutes. After differentiation with the Best's carmine differentiator for 5 minutes, they were washed in 80% alcohol and absolute alcohol. They were cleared in xylene and finally mounted in ucanparal.

4. **Alcian blue reaction** (Barka and Anderson, 1963): Zygotes and spores fixed in Carnoy's fluid and brought to water were stained for 10 minutes in 0.2% Alcian blue 8 GS buffered to pH 2.6. After 3 changes in distilled water and a brief rinsing in 1.0% acetic acid and another rinse in distilled water, the preparations were dehydrated and mounted in Canada balsam.
5. **Toluidine blue reaction** (Barka and Anderson, 1963): Smears of zygotes and also of spores fixed in Carnoy's fluid were washed in absolute alcohol and subsequently brought to water. They were then stained for 20 minutes with 0.1% toluidine blue prepared in 30% alcohol. After a brief rinsing in 95% alcohol and dehydration in absolute alcohol, the preparations were cleared in xylene and finally mounted in Canada balsam.

C. **Lipids**

1. **Sudan black B** (McManus, 1946; cited in Pearse, 1961): Zygotes and spores were fixed in calcium formol for about 30 minutes. After a thorough washing in distilled water and up-grading to 70% alcohol, the smears were stained in saturated Sudan black B stain for 30 minutes. They were then briefly rinsed in 70% alcohol and immediately washed in running water for 10 minutes, and finally mounted in glycerol jelly.

2. **Oil Red O** (Lillie, 1944; cited in Pearse, 1961): Formalin fixed zygotes were brought to water. After a thorough washing, they were stained in freshly filtered Oil Red O solution (0.5%), prepared in 98% isopropanol, for about 30 minutes; then they were briefly differentiated in 60% isopropanol, washed in water and mounted in glycerol jelly.
3. **Pettrot 7B method (Pearse, 1968):** The zygotes were fixed in 4% formalin. After thoroughly washing in water, they were upgraded to 50% alcohol and stained in a saturated alcoholic solution of Pettrot 7B for 15 minutes. After washing again in water the material was mounted in glycerol jelly.

4. **Nile blue method (Cain, 1947; cited in Pearse, 1961):** Zygotes fixed in calcium formol were washed thoroughly in water and stained with 1.0% aqueous Nile blue at 60°C for 5 minutes, washed in water, differentiated at 60°C only and finally they were mounted in glycerol jelly.

D. **Action of the fore-gut medium of the beetles** *Opatrum* sp. and *Mesomorphus velliger* on the spores of *S. conoides*

Mature spores of *S. conoides* were taken on clean heat-sterilized slides and a few drops the fore-gut fluid contents of two beetles *Opatrum* sp. (natural host of *S. conoides*) and of *Mesomorphus velliger* were separately added. Immediately the preparations were covered with coverslips and were observed under a binocular microscope.
Freshly formed zygotes of *Stylocephalus conoides* are colourless, oval shaped, transparent bodies. They are held with one another laterally in long chains. A tough coat is developed around each zygote about 10-12 hours after its formation. The zygote, with an extra coat around it, is known as a spore. The spore is a hat-shaped body, measuring 14.0 x 7.0 microns in size. Gradually the spores become grey, and by about another 12 hours they become dark grey (Fig. 23).

**Zygotes or early spores:**

**Localization of proteins:** Zygotes or immature spores stained with Bromophenol blue showed a mild blue colouration throughout (Fig. 24). A similar colouration was seen throughout the sporoplasm in 12 hours old spores in which the sporozoites were not yet formed. This suggested a uniform distribution of proteins in general in the zygotes and sporoplasm of early spores. In spores older than these, the results of bromophenol blue staining could not be observed properly owing to an increased pigmentation in the spore-coat.
A faint β-metachromasia with toluidine blue was seen in the nuclei of the zygotes showing the localization of nucleoproteins.

Localization of carbohydrates

With Best's carmine and with Lugol's iodine stains, the reaction was very faint in the wall of the zygotes (Fig. 25), which showed the presence of traces of carbohydrates. The reaction in the cytoplasm was almost negative. However, a few Lugol's iodine +ve purple brown coloured granules, the glycogen granules, were found in 12-24 hour old spores. Gradually, these granules considerably increased in number, and were spread throughout the sporoplasm in 36 hour old spores (Fig. 26). They were still very prominent in 48 hour old spores; however, the glycogen granules decreased in number in 60 and 72 hour old spores.

A faint β-metachromasia with toluidine blue and a light green colouration with alcian blue reaction found in the wall of the zygotes (immature spores) demonstrated the presence of traces acid mucopolysaccharides. However, similar colourations could not be discerned with certainty in the wall of the mature spores (24 hours of development and further) as the spore wall contained black pigment.
Localization of lipids:

The freshly formed zygotes showed a mild blue colouration in the wall and in the general cytoplasm with Sudan black B indicating the presence of lipids in general. There were 8–10 large SBB+ve, lipid droplets scattered in the cytoplasm (Fig. 27). These droplets stained bright red with Oil Red O and Fettrot 7B, while they stained purple or pink with Nile blue stains. In 12–36 hour old spores these SBB+ve droplets decreased in number (Fig. 28). In the 48 hour old spores the SBB reaction was in traces, whereas it was not found in 72 hour old spores, revealing the gradual depletion of the lipids in spores older than 48 hours.

Tests for the chemical composition of the 24 hour old spore coat gave the following results:

The spore coat gave a deep pink colouration with Performic acid–Schiff reaction (Fig. 29) and magenta colour with Ninhydrin reagent (Fig. 30); it stained light blue with Sudan black B (Fig. 27) suggesting its lipo-proteinaceous nature. The spore coats (capsules) did not dissolve in boiling H₂O, in dilute HCl and in dilute KOH, which suggested the presence of keratin and the absence of collagen in the
spore coat. With xanthoproteic reaction the spore coat did not develop any yellow colour, confirming thus the absence of collagen. Similarly Gram's iodine reaction also did not show any reddish violet colour indicating the absence of chitin in the spore coat. On the contrary, Masson-Fontana reaction produced an orange colour which was highly masked by the black colour (Fig. 31) confirming the presence of keratin type of protein in the spore coat.

Action of conc. HNO₃ on the spore wall first produced a yellow colour in it, which intensified on addition of a drop of conc. KOH (Fig. 32) revealing the presence of yet another protein viz., tyrosine which is the precursor of the pigment melanin. Results of the bleaching reactions were also positive. The black pigment of the spore-wall was completely bleached within five to ten minutes with peracetic acid (Fig. 33), while the bleaching action with H₂O₂, though complete, was much slower and required about 2 hours. These results of the bleaching reactions showed that the pigment in the spore-coat was melanin.

The results of the actions of the fore-gut fluids of two different beetles on the spores of S.conoides are given below.
All the spores which were exposed to the gut fluid of *Opatrum* sp. dehisced immediately or within a few minutes liberating the actively moving sporozoites (Fig. 34); on the contrary, none of the spores exposed to the action of the gut fluid of *Mesomorphous velliger* dehisced at all even after 1-2 hours of treatment (Fig. 35).

**DISCUSSION**

From the literature, it is evident that the structural properties and the metabolic activities within the spores have been studied in details only in some coccidians, *Eimeria tenella* (Gill and Ray 1954); *E. brunetti* and *E. acervulina* (Fattillo and Becker 1955); *E. necatrix*, *E. melea*, *E. grimitis* and *E. acervulina* (Vetterling and Doran, 1969) and *E. steidae* (Frandsen, 1970); *E. acervulina* (Smith and Long, 1963) and *Sarcocystis* sp. (Fayer and Thompson, 1975), and in different myxosporidians (Podlipaev 1972 and 1974). Podlipaev and Schulman 1973 and Donec *et al.* (1978). There are only three reports on similar studies on the zygotes of gregarines *Geneiorhynchus aescnæ* and *Hoplorhynchus oligoanthus* (Stein, 1961), *Enterocystis ensis* (Bobyleva, 1963) and *Diplocystis* spp. (Loubes and Bouix, 1970).
Opinions on the nature of the spore coat as well as the metabolic reserves in the spores of the sporozoan parasites are varied. Gill and Ray (1954) demonstrated the presence of acid mucopolysaccharides of hyaluronic acid type in the wall of the oocysts of Eimeria tenella, whereas Smith and Long (1963) found the presence of largely a lipo-protein or glycoprotein. Pattillo and Becker (1955) did not obtain positive results for the presence of lipids in the oocysts wall of E. brunetti and E. acervulina. Stein (1961) has not mentioned anything on the nature of the wall of zygotes of the two gregarines studied by him. Our observations on the spore coat of S. conoides show that it is made up of largely a lipo-proteinous substance. The magenta colour developed in the spore wall with Performic acid-Schiff reaction and Ninhydrin reaction shows that one of the proteins present is keratin. Probably the presence of keratin in the spore wall makes the spores resistant to descication, temperature changes, radiation effects, microbial infections (Encyclopaedia Britanica Vol. VI, pp. 767, 1974). Further, the pigment melanin in the spore wall seems to prevent light rays from reaching the sporozoites in S. conoides.

There are also different opinions on the nature of metabolic reserves in different groups of sporozoa. Stein
observed the presence of paraglycogen (amylopectin) in the sporozoites of Geneiorhynchus and Hoplorhynchus. It is therefore implied that the spores of these gregarines contain paraglycogen. Similar findings have been reported by Bobyleva (1963) in Enterocystis ensis and by Loubes and Bouix (1970) in Diplocystis spp. Smith and Long (1963) reported the presence of a rich amount of lipids in the early oocysts of Eimeria acervulina, however, they found that the lipids were converted into carbohydrates during the later stages of sporulation. Frandson (1970) has also reported the presence of rich amount of lipids in the oocysts of E. steidae but has not said anything about the conversion of the lipids to carbohydrates. Vegterling and Doran (1969) found both lipids and the carbohydrate—amylopectin in the oocysts of E. acervulina, E. crimitis, E. necatrix and E. meleag. Similarly, Podlipaev (1972 and 1974) observed lipids and glycogen in the early stages of oocysts of various species of Myxobolidae (Myxosporidiae; Sporozoa). He further stated that in the sporulating stages the number of glycogen granules increased, and that these granules were stored in a definite vacuole called iodinophilous vacuole (Podlipaev 1974). Gradually this stored polysaccharide passed into the sporoplasm. The vacuole thus serves as a depot of reserve nutrients for
the spores of *Myxobolidae* (Podlipaev and Schulmar 1978).

Donee et al. (1978) identified two types of spores of myxospordians which are the gill parasites of fresh-water fishes; one type containing the iodinophilous vacuole, and the other type lacking it. The former type is produced in genera *Myxobolus*, *Myxosoma* and *Thelohanellus* being loaded with glycogen and hence heavier than the other type of spores, sinks in the waters; these spores thus easily get access to the gills of benthophagous and phytophagous fishes. The spores, which almost lack glycogen, are produced in genera *Myxidium*, *Henneguya*, *Myxobilatus*, *Chloromyxum*, *Hoferells*, and *Sphaerospora*; being relatively lighter, these spores float in waters and can get an access to the gills of the surface dwelling planktophagous fish. Thus the authors find that the iodinophilous vacuole in the myxospordians plays a role in the vertical dispersion of spores depending upon whether these spores infect the gills of the surface or benthic feeding fishes. Payer and Thompson (1975) observed numerous small lipid droplets as well as PAS-ve granules in the oocysts of *Sarcocystis* sp.

Our findings on the zygote-spore contents in *S. conoides* are in agreement with those of Horton-Smith and Long (1963) in that the lipids are the main metabolites
during early stages of sporulation i.e. the zygotes, and, in the later stages, i.e. the spores, the carbohydrates are the major metabolites. Therefore, it seems reasonable to suppose that in *S. conoides* the lipid reserves of the early stages of the spores get converted into carbohydrates through glyoxalate cycle as the spores mature. This is a suggestion and needs further work for confirmation. Such a phenomenon was first observed in the bacterium *Pseudomonas* strain (Kornberg and Krebs 1957); Subsequently it has been observed also in the oocysts of *Eimeria tenella* (Nath and Dutta, 1962), in *E. acervulina* (Smith and Long, 1963) and in the ciliate *Tetrahymena* sp. (Hogg and Kornberg, 1963). Stein (1961) has reported the presence of a few polysaccharide granules in the sporozoites. Further, it seems that the metabolic shift in the spores, at least in *S. conoides*, is related to the formation of keratinaceous, resistant, impermeable spore coat which incidentally cuts off the oxygen supply to the sporozoites that are formed in the spores, and that the sporozoites are forced to resort to anaerobic metabolism. The basic material for this type of metabolism is a carbohydrate. It may be mentioned, in this context, that the activities of only lactate dehydrogenase (LDH) and $\alpha$-Glycerophosphate
dehydrogenase (α-GPDH) have been observed in the sporozoites of S. conoides (Chapter 4 in the present work).

Our observations on the action of the fore-gut fluid of two different beetle species, viz., Opatrum sp. and Mesomorphus velliger on the spores of S. conoides, reveal yet another interesting feature of the spores. The coat is resistant to the enzymic actions of the fore-gut fluid of any unnatural host. The spores dehisce instantaneously and liberate the sporozoites into the gut only when they are ingested by the natural hosts. Otherwise, they will neither dehisce nor their coat is dissolved; this property of the spore capsule ensures safety to the sporozoites under circumstances when some wrong hosts accidentally ingest the spores. Perhaps by this, the host-parasite specificity is also maintained to a large extent.
SUMMARY

1. Some cytochemical studies on the spores of the gregarine *Stylocephalus conoides* have been made in the present work.

2. The presence of keratin in the spore coat of the spores has been demonstrated. Probably because of this protein in its coat the spore becomes tough and resistant capsule protecting the sporozoites against desiccation, temperature variations, microbial infection and the action of the digestive enzymes of any unnatural host.

3. The dark pigment present in the spore wall of *S. conoides* has been shown to be melanin. This pigment in the spore wall may serve to cut off the light rays reaching sporozoites and continuously stimulating them; this pigment may also help in protecting these bodies from the harmful radiations.

4. Action of the fore-gut of the host on the dehiscence of the spores has also been studied in the present work. The spores will dehisce only when they are exposed to the fore-gut fluid of the natural host.
and not otherwise. This observation suggests a host-parasite specificity.

5. The immature spores (Zygotes) of *S. conoides* are rich in lipids but are devoid of polysaccharides. However, during the course of maturation, the lipids disappear and the polysaccharides (glycogen) appear; and the 48 hour old spores are found to be full of glycogen. This suggests a gradual shift from the lipid-dependent metabolism to the one depending on the carbohydrates.