CHAPTER IV

Electrophoretic studies on the soluble proteins of the primates and the satellites of the gregarine

_Hirmocystis incola_
INTRODUCTION:

Electrophoretic studies on protein fractions have been in a few protozoa, viz., Amoeba Sp. (Kates and Goldstein, 1964); in Plasmodium knowlesi (Williamson, 1967*), in Trypanosoma cruzi (Afchain and capron, 1969*), in Leishmania maxicana (Crook et al., 1969*). In Plasmodia, both macrogametogenesis and microgametogenesis are shown to be dependent on de novo protein synthesis (Toyé et al., 1977 and Kumar et al. 1983). In Plasmodium falciparum, incorporation of $^3$H] leucine (Phillips et al., 1978) and $^{35}$S] methionine (Simm, unpublished work cited in Sinden, 1983 a) into the gametocytes has been studied. However, the ultrastructural studies of 'avian', 'reptilian' and 'mammalian' plasmodia have revealed that the microgametocyte has a limited potential for de novo protein synthesis, when compared to the macrogametocyte (Aikawa et al., 1969 and Sinden, 1983, b). In Paramecium, it is shown that the mating activity is dependent on a soluble protein (Nobuyuki and Karina, 1986).

The qualitative and quantitative aspects of naturally occurring metabolites have been studied so far in a single gregarine species S. mesomorphi (Amoji, 1975). This may be due to the fact that they lack the sexual reproduction (Schizogony) in their life-cycle and hence they can not be cultured. In addition, they are relatively smaller organisms and hence cannot be isolated in sufficient numbers easily. However, the cytochemical studies using Mercuric Bromophenol Blue (MBB) technique have revealed the presence of protein in all the stages of development of a few gregarine species viz., G. aeshnae and

H. oligocanthus (Stein, 1961), P. crystalligera (Collins, 1972); S. mesomorphi (Amoji, 1975) and S. conoides (Desai, 1980).

The female sex-specific proteins (vitellogenins) have been identified in the haemolymph of several adult insects, (Englemann, 1970 and 1972) and a review, Docne, 1973). Such female sex-specific proteins have been identified by electrophoretic techniques in *Gryllus domesticus* (Kunz and Petzelt, 1970) and *Acheta domesticus* (Bradley & Edwards, 1978).

The above mentioned studies in *Plasmodia* and *Paramecia* indicate that some specific soluble proteins have some significant role in the sexual reproduction and gametogenesis in protozoans also. In the present study, an attempt is made to understand the qualitative and quantitative differences between the soluble proteins of parasite and satellite, of the gregarine *Hirmocystis incola*. Electrophoretic studies have been carried out to know whether there are any additional protein fractions comparable with female specific proteins observed in insect species, either in the parasite or the satellite.

**Materials and Methods:**

**Protein Estimation:**

The gamonts of *H. incola* in syzygies were collected from the host gut as described earlier as they are larger and easy to handle. The parasites and satellites were separated by a brush bristle, washed in distilled water and were transferred to two separate centrifuge tubes containing 1 ml of distilled water and kept in an ice bath. In one tube (P) 500 intact parasites and in another tube (S) their 500 satellites were collected. The two samples formed
one set, likewise four more sets of the gregarine samples were prepared for the present work. They were centrifuged and resuspended in 2 ml of 0.1 M sodium phosphate buffer (pH 7.6). They were preserved at -20°C till further work was carried out. The suspensions were sonicated in an ultrasonicator and centrifuged at 3000 rpm for 20 minutes. The total soluble protein content of the supernatant was estimated by adopting Lowry et al. (1951) method. The colour intensity was measured on Bausch and Lomb Spectronic 2000 U.V/vis spectrophotometer at 660 nm. Bovine serum albumin was used as a standard protein for calibration of the standard curve. The quantities of proteins of the primate and the satellite samples so obtained were assessed further by applying Student's 't' test to know whether there was any statistically significant difference between the two samples.

**Electrophoretic study:**

Soluble proteins of the primate and the satellite were analysed by sodium dodecyl Sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli, (1970). Cylindrical, 10% Polyacrylamide gels of 7.5 cm length were used for electrophoresis.

The gel composition was as follows:—

1) Running Gel.

1 gm Acrylamide.

26.7 mg NN' Bis acrylamide.

0.0025 ml TEMED.

2.5 mg of Ammonium persulphate in a total volume of 10 ml.

of 0.375 Tris HCL (pH 6.8) containing 0.1% Sodium dodecyl sulphate.
2) **Stacking gel:**

- 300 mg Acrylamide.
- 8.0 mg NN' Bis acrylamide.
- 0.0025 ml TEMED.
- 2.5 mg Ammonium per sulphate in a total Volume of 0.125 Tris HCl (pH 6.8) containing 0.1% SDS.

3) **Tank buffer:**

- Tris - glycine buffer pH 8.3
  - 302.5 mg Tris.
  - 1.441 gm glycine
  - 100 mg SDS in 1000 ml of H2O

4) **Sample buffer:**

- 0.0625 Tris - HCl. containing 2% SDS and 10% glycerol.

**Sample preparation:**

From the supernatant obtained as mentioned earlier, 0.5 ml was mixed with 0.5 ml of the sample buffer and kept in boiling water bath for 5 minutes. 0.3 ml of this aliquot for both samples were loaded for electrophoretic run. Electrophoretic run was carried out for one hour using 3.5 m.amps per tube simultaneously at room temperature.

**Fixation and Staining:**

Gels were fixed overnight in 3-4 volumes of 50% Trichloroacetic acid (TCA) and stained with Coomassie brilliant blue R-250, (1.25 gm. in a mixture of methanol; glacial acetic acid and water in ratio 400 : 70 : 530 by volume) at room temperature. Destaining
was carried out in an agitator containing 7.5% acetic acid.

**Molecular weight determinations:**

The molecular weights of proteins were estimated by SDS-PAGE using standard marker proteins. The below mentioned marker proteins were used for obtaining the calibration curve - lysozyme - 14,300; β-lactoglobulin - 18,400; trypsinogen-24,000; pepsin-34,700; ovalbumin-45,000 and bovine serum albumin - 66,000.

The mobility of standard marker was plotted against log. mol. wt.. From this calibration curve, the mobilities of the separated fractions were compared with standard proteins.

Mobility of the protein fractions of the samples was calculated as follows:

\[
\text{Distance moved by the protein fraction} \div \text{Length of gel before staining} \times \frac{\text{Length of gel after destaining}}{\text{Distance of the tracking dye Moved}}
\]

The sample gels were scanned with Shimadzu CS-910, Dual wave length TLC Scanner at 560 nm. The areas of absorbance peaks were calculated by the number of squares in the graph.

**Results:**

The quantitative estimations have revealed that the total soluble proteins of the primite sample ranged from 49 to 51 μg/0.5 ml at an average of 50.2±0.34 μg/0.5 ml pooled out from 500 primites; likewise, the total soluble proteins of the satellite ranged from 47
to 49 μg/0.5 ml at an average of 48.0±0.316 μg/0.5 ml of the sample pooled out from 500 satellites. The amount of soluble proteins was more in the primites than in the satellites. This difference in the quantity of soluble proteins is highly significant (P < 0.001).

**Results of SDS-Polyacrylamide gel electrophoresis:**

Five similar major protein fractions have been identified in both the primites and the satellite samples (Fig. 46). Absorbance scans of the stained gels also confirmed the presence of only five major protein fractions in both the primites and satellites i.e. fraction, I, II, III, IV, and V (Fig. 47a and b). The total area occupied by the protein fractions of the primites was 1493 mm² and the total area occupied by the protein fractions of the satellites was 550 mm². The variations in the areas occupied by each of the absorbance peaks were also found. The areas occupied by the individual protein fractions of the primites were 218 mm², 314 mm², 552 mm², 216 mm² and 223 mm², respectively for the fractions, I, II, III, IV, and V (Fig. 47a and b). The areas occupied by the individual protein fractions of the satellites were 136 mm², 66 mm², 399 mm², 101 mm², and 148 mm², respectively for the protein fractions, I, II, III, IV, and V. The areas occupied by the protein fractions of the primites were found to be more than those of the satellite protein fractions.

The molecular weights of the soluble protein fractions of the primites and satellites were, 56,200, 45,703; 25,000; 19,000; and 15,330, respectively for, I, II, III, IV, and V fractions. (Fig. 48. and Table, X).
Discussion:

In the unfractionated soluble protein extracts of *Amoeba proteus* Bk strain, *A. proteus* t strain and *A. discoides*, 18 components and in *Chaos chaos*, 22 components have been observed (Kates and Goldstein, 1964). Further the authors regard the aforesaid three amoeba species as one and the same on the basis of their electrophoretic studies. Similarly, trypanosomes of *bruci* group are shown to contain at least 22 components (Nojugu and Humphreys, 1967). *P. knowlesi* is shown to contain six main groups of soluble proteins and at least 11 precipitogens (Williamson, 1967). The culture form *T. cruzi* is shown to contain at least 19 components (Afchain and Capron, 1969) and *L. maxicana* is shown to contain 11 components. To the best of our knowledge no such studies have been carried out in the gregarines so far. We have observed 5 major soluble protein fractions in the gregarine *Hirmocystis incola*.

In *Plasmodium yoelii*, it is shown that for microgametogenesis de novo protein synthesis is essential and that the major proteins are synthesised during microgametogenesis (Toyé *et al.*, 1977). Further, it is shown that in *P. gallinaceum* macrogametogenesis is not inhibited by actinomycin D, but it is blocked by the inhibitors of protein synthesis indicating the de novo protein synthesis during macrogametogenesis (Kumar *et al.*, 1983). It is also reported that the uptake of $[^3H]$ leucine into the gametocytes of *P. falciparum* is less rapid than the erythrocytic schizont (Phillips *et al.*, 1978). Similarly, the uptake of $[^35S]$ methionine into stage III and V gametocytes have been studied and it is revealed that 26 individual proteins are synthesised at this

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stage, of which 6 are unique to the sexual parasites, i.e. gametocyte and the other proteins are similar to those of sexual schizont, i.e. a stage intermediate between the undifferentiated schizont and the gametocyte (Simm, Unpublished work cited in Sinden 1983a). The ultrastructural studies have revealed that irrespective of 'avian', 'reptilian' and 'mammalian' sub grouping the male and female gametocytes of Plasmodia are of highly conserved morphology (Aikawa et al., 1969 and Sinden, 1983b). Further, the female/macrogametocyte has a small nucleus and the cytoplasm contains extensive endoplasmic reticulum, numerous mitochondria, more ribosomes and numerous small membrane bound osmophilic bodies. The male/microgametocyte contrasts with the female in having a large octoploid, digitate nucleus and its cytoplasm contains relatively a few osmophilic bodies and poorly developed endoplasmic reticulum, suggesting a limited potential for de novo protein synthesis (Aikawa et al., 1969 and Sinden, 1983b). In Paramecium, it is reported that the decrease in the sexual activity caused by aging is not due to defects in the genes for the mating substance but due to decrease in the activity of a soluble protein, which is responsible for the expression of mating activity. The factor responsible for the recovery of the sexual activity is shown to have the same characteristics as immaturin in terms of molecular size and net charge (Nobuyuki and Karina, 1986). These findings indicate that the proteins including the soluble proteins have significant role in the sexual reproduction in protozoans also. Further they also indicate the occurrence of de novo protein synthesis during gametogenesis.
The cytochemical studies using MBB technique have revealed the presence of proteins in the cytoplasm of all the stages of the life-cycle of a few gregarines such as *G. aeshnae* and *H. oligocantus* (Stein, 1961); *P. cryfalligera* (Collins, 1972); and *S. conoides* (Desai, 1980). In *S. mesomorphi*, it is revealed that the lipids on wet basis constitute relatively high percent i.e. 28.45 when compared to total proteins 1.83; soluble proteins 0.6 and carbohydrates 3.10 (Amoji, 1975).

In the present study, the quantitative estimations have revealed that the primates contain more amount of soluble proteins than the satellites in *H. incola*, and the difference is statistically significant.

The female sex-specific yolk proteins (*Vitellogenins*) have been identified in the haemolymph of several adult insects (Engleman, 1970 and 1972 and a review, Doane, 1973). It is shown that these female sex-specific yolk proteins can be detected by electrophoretic studies in the haemolymph of mature females and their absence in the haemolymph of mature males of the insects *Gryllus domesticus* (Kunz and Petzelt, 1970) and *Acheta domesticus* (Bradley and Edwards, 1978). In *Gryllus domesticus*, using cellulase acetate gel electrophoresis, a sex-specific yolk protein has been identified (Kunz and Petzelt, 1970) and in *Acheta dometicus*, using SDS-PAGE four female sex-specific yolk proteins have been identified (Bradley and Edwards 1978).

In the present SDS-PAGE study of soluble proteins of the primates and the satellites of the gregarine *H. incola*, no such extra protein fractions have been observed. We have observed similar 5 major protein fractions in both the primates and the satellites. However,
variations in the total areas of the absorbance peaks of different frac-
tions of the primites and the satellites are observed. The total area
of absorbance of the primites is found to be more than that of the
satellites, which is similar to the quantitative differences observed
in the estimations.

In plasmodia, both microgametogenesis and macrogametogenesis
are shown to be dependant on de novo protein synthesis (Toyé, et
al., 1977 and Kumar et al., 1983). Further, the ultrastructural studies
have shown that the microgametocytes of plasmodia have limited poten-
tial for protein synthesis than the macrogametocytes (Aikawa et al., 1969 &
Sinden, 1983). Our findings indicate that the primites of H. incola contain
more amount of soluble proteins than the satellites. Hence the primites
may be assigned with the femaleness and the satellites with maleness.
However, these findings in H. incola need additional support from the
ultrastructural and further biochemical studies. As such the findings
cannot be generalised to all the gregarine species. Such studies should
be carried out on some more species to corroborate or modify our
view. To elucidate the role of proteins in the sexual differentiation
and gametogenesis in gregarines further studies on proteins at different
stages of the life cycle of gregarines are needed.
SUMMARY

1. The quantitative estimation of water soluble proteins of the pnmites and the satellites of the gregarine *Hirmocystis incola* in gamont condition and also their Sodium Dodecyl Sulphate (SDS) gel electrophoretic study have been carried out.

2. The quantitative studies have revealed that the pnmites of *H. incola* contain more amount of soluble proteins than the satellites and the difference in the quantity is found to be statistically significant.

3. SDS gel electrophoretic studies have revealed that, both the pnmites and the satellites of *H. incola* have the similar five major soluble protein fractions.

4. There seems to be some inexplicable biochemical differentiation at the protein level also in the pnmites and the satellites of *H. incola*.

5. The pnmites containing more amount of soluble proteins may be assigned with femaleness and the satellites with lesser amount of soluble proteins may be assigned with maleness.
### TABLE- X

Showing the mobility observed; true mobility, log. Mol. Wts. of the standard proteins and the protein fractions, I, II, III, IV and V of the primites and the satellites of the gregarine _d. incola._

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<td>B.S.A(1)</td>
<td>1.4</td>
<td>0.2632</td>
<td>4.8195</td>
<td>66,000</td>
<td>2.0</td>
<td>0.3040</td>
<td>4.750</td>
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<td>Albumin (2)</td>
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<td>0.3952</td>
<td>4.660</td>
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<td>Pepsin^- (3)</td>
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<td>0.4136</td>
<td>4.6166</td>
<td>34,700</td>
<td>4.3</td>
<td>0.6536</td>
<td>4.410</td>
<td>25,700</td>
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<tr>
<td>Trypsinogen (4)</td>
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<td>B-Lactoglobulin(5)</td>
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<td>0.8084</td>
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<td>0.7904</td>
<td>4.280</td>
<td>19,000</td>
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<tr>
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<td>4.1553</td>
<td>14,300</td>
<td>5.8</td>
<td>0.8816</td>
<td>4.1850</td>
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