CHAPTER VI

STUDIES ON THE CYTOCHEMICAL DEMONSTRATION OF SOME STEROID DEHYDROGENASES IN THE GREGARINE STYLOCEPHALUS CONOIDES DEV.
INTRODUCTION:

Biosynthesis of steroids and their in vitro conversion have been studied recently in several invertebrate metazoans, viz., echinoderms, molluscs, crustaceans, insects and nematodes (Briggs, 1972; De Longcamp, 1974; Sandor, 1975 -- a review; and Gouder et al., 1977). Likewise, steroids and sterols have also been reported in a few protozoans other than sporozoans (Sebek and Michaels, 1957; Conner et al., 1966; and reviews: Wagtendonk, 1955; Wright, 1964; Charney and Herzog, 1967; Dewey, 1967 and Shorb, 1967). However, these studies have not been extended to at least a few species of sporozoans. The presence of cholesterol has been reported in only one species of gregarine Stylocephalus mesomorphi (Amoja, 1975). It may be pointed out here that all these studies on protozoans are confined to the trophic forms which represent the nutritive and growing stage. Therefore it would be interesting to investigate the presence/absence of some enzymes involved in the biosynthesis of steroids in different reproductive stages also of a protozoan, a gregarine in the present work.
Sebek and Michaels (1957) have biochemically demonstrated the in vitro conversion of steroids in *Trichomonas foetus*, *Trichomonas gallinae* and *Euglena gracilis*, which is similar to the steroid conversion found mammalian tissues and various microorganisms. Wright (1964) from his studies on *Entamoeba coli*, reported that this organism is capable of synthesizing sterols de novo. Conner *et al.* (1966) observed the conversion of cholesterol into cholesta-5, 7, 22-triene, 3β-OH in the ciliate *Tetrahymena pyriformis*. On the other hand, Britt and Bloch (1961) recovered the tritiated lophenol, practically unchanged from the medium after the growth of *Tetrahymena corlissi*. Hutner (1964) believed that the protozoans, whether free-living or parasitic, do depend upon the exogenous supply of sterols. Gutteridge and Coombs (1977) opined that most of the parasitic protozoa are incapable of steroid synthesis and hence must procure them from the ambient medium. The brief review of the literature suggests that it is not certain whether protozoans are capable of synthesizing steroids or not.

A cursory survey of the literature reveals that there is a need to carry out further work to find whether protozoans (including their reproductive stages) have the potential for steroid biosynthesis or not. Therefore an
attempt is made in the present study to carry out the histochemical identification of, a) Lipids, b) Cholesterol and its esters and c) \( \Delta^5 \)-3\( \beta \)-hydroxysteroid dehydrogenase (\( \Delta^5 \)-3\( \beta \)-HSDH), 17\( \beta \)-hydroxysteroid dehydrogenase (17\( \beta \)-HSDH), 11\( \beta \)-hydroxysteroid dehydrogenase (11\( \beta \)-HSDH) and glucose-6-phosphate dehydrogenase (G-6-PDH) in different stages of the life cycle of the gregarine Stylocephalus conoides. The presence of lipids, cholesterol and a given dehydrogenase/s in an organism has been taken as an evidence of its steroidogenic potential.

MATERIALS AND METHODS

The trophozoites and gamonts of Stylocephalus conoides obtained from freshly collected host beetle Opatrum sp. were used in the study. Similarly, smears of gametes and zygotes, as well as the squash preparations of sporozoites were also used in the present work.

A) Demonstration of lipids:

After fixing in calcium formol for 15 minutes the trophozoites, gamonts, gametes, zygotes and sporozoites were washed thoroughly in distilled water. This was followed by staining with Sudan black B, Oil Red O, Pettrot 7B and Nile blue methods for the demonstration of neutral lipids.
Demonstration of cholesterol and its esters

Some trophozoites and gamonts were examined for the presence of cholesterol and its esters following the

1) Schultz method and ii) Okamoto method.

i) Schultz Method (after Weber et al., cited in Pearse 1961):

Specimens fixed in calcium formol were washed in distilled water for 24 hours (several changes) and treated for seven days at 37°C with 2.5% ferric ammonium sulphate prepared in 0.2M acetate buffer (pH 3.0). They were washed in 3 changes of acetate buffer, 1 hour each, and then with distilled water before they were treated for 10 minutes with 5.0% formalin. The specimens were taken on a slide and were placed in a drop of a mixture of conc. H₂SO₄ and acetic acids and were covered with a cover slip. The preparations were examined for the development of greenish blue colour.

ii) Okamoto method (after Udea; cited in Pearse, 1961):

Trophic forms of S. conoides mounted on a slide were fixed in 4% formalin for 15 minutes and were thoroughly washed in distilled water. The water was removed by blotting paper and they were treated with 2-3 drops of freshly
prepared sulphuric iodine (conc. $\text{H}_2\text{SO}_4$ 15 ml + alcoholic iodine 2 ml). Alcoholic iodine was prepared as follows:

Ethyl alcohol 95% 1.8 ml. + Iodine 65 mg.  
+ Potassium iodide 25 mg.

The preparations, after applying the coverslips, were examined under the microscope for the development of bluish green colour.

C) Demonstration of steroid dehydrogenases:

The trophic forms, gametes, zygotes and sporozoites were frozen over dry ice vapours at about $-50^\circ\text{C}$ and immediately thawed at room temperature; after a brief rinsing in cold acetone ($4^\circ\text{C}$), the preparations were incubated in appropriate media under aerobic conditions 10-30 minutes. They were fixed in neutral formalin, washed in water and mounted in glycerol jelly.

For the demonstration of hydroxysteroid dehydrogenases, Wattenberg's method (1958) modified by Levy, et al., (1959) and subsequently modified by Baillie et al. (1966) was adopted. The detailed procedure is already described by our previous workers in laboratory (Saidapur and Nadkarni, 1972).
The incubation medium consisted of the steroid substrate 1 mg/ml. dissolved in DMP, the co-enzyme NAD 1.5 mg/ml., the hydrogen acceptor NBT (Nitro Blue Tetrazolium) salt 1 mg/ml. and 0.2M phosphate buffer (pH 7.4).

The following substrates were used for the histochemical demonstration of a) \( \Delta^5 \)-3\( \beta \)-HSDH, b) 17\( \beta \)-HSDH and c) 11\( \beta \)-HSDH activities.

a) \( \beta \)-HSDH activity:

1. Pregnenolone
   \((3\beta\)-hydroxypregnen-5-20-one\)
2. Dehydroepiandrosterone (DHEA)
   \((3\beta\)-hydroxyandrost-5-ene-17-one\)

b) 17\( \beta \)-HSDH activity:

1. 17\( \beta \)-estradiol
   \((3, 17\beta\)-dihydroxy-1,3,5(10)-estratriene\)
2. Testosterone
   \((17\beta\)-hydroxyandrost-4-en-3-one\)

c) 11\( \beta \)-HSDH activity:

11\( \beta \)-hydroxyandrostenedione
\((11\beta\)-hydroxyandrost-4-en-3, 17-dione\).
Some trophozoites were incubated under identical conditions in a substrate-free medium. A second sample of trophozoites was treated for 10 minutes with cyanoketone dissolved in a few drops of 0.2M phosphate buffer (pH 7.4) and was then incubated in the normal incubating medium containing the substrate Dehydroepiandrosterone (DHEA). The first sample served as the control for the hydroxysteroid dehydrogenase activity in general, while the second sample served as the control specifically for 3β-HSDH activity.

D) NADH diaphorase activity:

The incubation medium used for the demonstration NADH diaphorase activity consisted of reduced NAD (NADH) 0.5 mg/ml. and NBT 0.5 mg/ml. dissolved in 0.2M phosphate buffer (pH 7.2).

E) Glucose-6-phosphate dehydrogenase (G-6-PDH) activity:

For the demonstration of Glucose-6-phosphate dehydrogenase activity, the method of Cohen (1959) modified by Saldapur and Nadkarni (1972) was adopted. The incubation medium consisted of D-Glucose-6-phosphate disodium salt (1.5 mg/ml), the coenzyme NADP (nicotinamide adenine dinucleotide phosphate) 0.5 mg/ml. and NBT (Nitro Blue
Tetrazolium) salt 0.5 mg/ml. dissolved in 0.2M phosphate buffer (pH 7.2). The intensities of the enzyme reactions were subjectively graded as follows:

1. **Maximum activity** = ++++
2. **Minimum activity** = +
3. **Traces of activity** = ±
4. **No activity** = −

Sigma grade fine chemicals obtained from the Sigma Chemical Company, U.S.A. were used. The observations are shown in Table-8 and supported by some photomicrographs.

**OBSERVATIONS**

1. **Lipids and Cholesterol:**

   The trophozoites, gamonts, gametes and zygotes of *Stylocephalus conoides*, showed a rich distribution of Sudan black B +ve droplets. The sporozoites did not show any SBB +ve droplets. In material tested for Nile blue reaction, the pink or purple coloured droplets were densely distributed; the sporozoites did not show droplets of any colour; with Oil Red O and Fettrot 7B, the red coloured droplets were seen throughout the body of trophozoites, gamonts,
gametes and zygotes (Chapter I). In trophozoites tested for Schultz reaction a greenish blue colour was noticed in the deutomerite region; similarly with Okamoto reaction a bluish green colour developed only in the deutomerite region. In gametes and zygotes the bluish green colour was developed with Okamoto reaction but it was very faint and temporary.

2. $\Delta^5-3\beta$-HSDH:

Diformazan granules, indicating the presence of $\Delta^5-3\beta$-HSDH, were seen throughout the body of the trophozoite (Fig. 105). The granules were somewhat concentrated in the deutomerite. Similarly, the granules were also concentrated around the nuclear wall. In trophozoites incubated in the substrate-free medium the formazan granules were not seen (Fig. 106); likewise, the diformazan granules were not seen in the trophozoites treated with cyanoketone prior to their incubation. Diformazan granules were also not seen in the gametes; but they were distinct and scattered throughout the zygotes (Fig. 107). The sporozoites, on the other hand, did not show the diformazan granules (Fig. 108).

3. $17\beta$-HSDH:

The presence of diformazan granules formed the basis of indication of $17\beta$-HSDH activity. The bluish black
granules were seen throughout the body of the trophozoite (Fig. 109). They were more concentrated in the protomerite than in the deutomerite. The granules just beneath the ectoplasm were larger than those found deep in the endoplasm. Around the nuclear wall the diformazan granules were more concentrated than elsewhere. Gametes and sporozoites did not show any diformazan granules whereas the granules were found in the zygotes (Fig. 110).

4. 11β-HSDH:

The diformazan granules were more densely packed than those for 17β-HSDH (Fig. 111). However, as in the case of 17β-HSDH activity, the granules were larger just beneath the ectoplasm than those in the deeper portions and they were conspicuously packed around the nucleus. The gametes and sporozoites yielded negative results. The formazan granules were uniformly distributed throughout the zygotes (Fig. 112).

5. G-6-PDH:

An intense activity was seen throughout the body of the trophozoite (Fig. 113). The diformazan granules were large in the protomerite but less densely distributed, whereas they were small and densely distributed in the
deptomente. The gametes and zygotes also contained the diformazan granules (Figs. 114 and 115). The sporozoites showed only traces of G-6-PDH activity.

6. NADH-diaphorase:

The activity of this enzyme in different stages of S. conoides has been described in chapter 4 in the present work.

DISCUSSION

Sandor (1975) has reviewed the work done on the occurrence of steroids and steroid dehydrogenases in several invertebrate groups. The occurrence of steroids and sterols in different species of protozoa, free-living as well as parasitic, has been reviewed by Wagtendonk (1955), Wright (1964), Dewey (1967), Shorb (1967) and Charney and Herzog (1967). The species studied are Entamoeba histolytica, Amoeba sp., Euglena sp., Trichomonas spp., and Paramoecium aurelia. The sterols and steroids from these organisms are cholesterol, stigmastenol, β-stigmasterol, gluco-corticoids, testosterone and progesterone. Eventhough some representative species from Rhizopoda, Mastigophora and Giliata have been studied by earlier workers, surprisingly no representative from Sporozoa has been examined so far but for a single report on the presence of cholesterol in the gregarine Stylocephalus mesomorphi (Amoji, 1975). In our present work,
besides the presence of cholesterol, three steroid dehydrogenases, viz., $\Delta^5$-3$\beta$-HSDH, 17$\beta$-HSDH, and 11$\beta$-HSDH have been reported in the trophic stages and zygotes of the gregarine *Stylocephalus conoides*.

Sebek and Michaelis (1957) have demonstrated the in *vitro* conversion of steroids in *Trichomonas vaginalis* and *Buglana gracilis* in a manner similar to those in mammalian tissues and various microorganisms. Wright (1964) has shown that *Entamoeba coli* has the ability to incorporate the radioactive stigmasterol which suggest its ability of sterol biosynthesis. Conner et al. (1966) have shown that the ciliate *Tetrahymena pyriformis* has the ability to convert cholesterol into cholesta-5,7,22-triene, 3$\beta$-ol.

On the other hand, Brit and Bloch (1961) recovered tritiated lophenol totally unchanged from the culture medium of *Tetrahymena corlissi*. Similarly, the anaerobically grown yeasts, some parasitic trichomonads, *Paramoecium* spp. and *Tetrahymena* spp. are known to depend upon the exogenous supply of steroids (Hutner, 1964). Gutteridge and Combs (1977) believe that most of the parasitic protozoa cannot synthesize steroids and as such they have to procure these substances from the external environment.
Amoji (1975) has reported the presence of free fatty acids, cholesterol and NADPH in the trophozoites of Stylocephalus mesomorphi. We have also observed a rich amount of neutral lipids and cholesterol in the trophozoites of S. conoides. In addition, certain key enzymes involved in the biosynthesis of steroids, viz., 3β-HSDH, 17β-HSDH and 11β-HSDH, are also reported in S. conoides in the present work. All these findings together suggest that the gregarine S. conoides has the potential to synthesize the steroids. However, further work, i.e., demonstration of steroid conversion, in vivo or in vitro, is necessary to confirm the view.

In vertebrate tissues, some structural aspects of cells are often correlated with steroid synthesis. It is reported that steroidogenic cells in the gonads of vertebrates are characterized by the presence of smooth endoplasmic reticulum and spherical mitochondria with tubular cristae (Christensen and Gillim, 1969). Similar mitochondria have been described in Pelomyxa carolinensis (Reuben, 1955), Trichomonas muris (Anderson, 1955), Amoeba proteus (Grieder et al., 1958), Mastigophora locustae (Harry and Finlayson, 1976) and in the gregarine Didymophyes gigantes (Hildbrand, 1976). These structural peculiarities suggest that the
protozoans may have the steroidogenic potential. However, this view is subject to further confirmation by additional studies.

\( \Delta^5 \)-3\( \beta \)-HSDH is known to be involved in the early biosynthesis of steroids and its presence has been histochemically demonstrated in all the steroidogenic tissues of vertebrates (Sameuls et al., 1951 and Baille et al., 1966). This enzyme is known to bring about the bioconversion of \( \Delta^5 \)-3\( \beta \)-hydroxysteroids into \( \Delta^4 \)-keto-steroids in the presence of an isomerase. It catalyses the conversion of pregnenolone to progesterone, 17\( \beta \)-OH pregnenolone to 17\( \beta \)-OH progesterone and DHEA to androstenedione. Therefore the presence of this enzyme, with DHEA as the substrate used in the medium in the present work suggests that the gregarine Stylocephalus conoides has the enzyme necessary to convert DHEA into androstenedione.

17\( \beta \)-HSDH is another essential enzyme involved in the biosynthesis of sex steroids, the androgens and estrogens. This enzyme catalyses the interconversions of testosterone = androstenedione and 17\( \beta \)-estradiol = estrone. Our histochemical demonstration of 17\( \beta \)-HSDH in S. conoides using both the substrates, testosterone and 17\( \beta \)-estradiol, might suggest the presence of both the steriospecific 17\( \beta \)-HSDH in this
gregarine. The presence of 17β-HSDH in *S. conoides* suggests that the gregarine has the potential to synthesize the sex steroids. It might be recalled here that Wright (1964) has observed an aggregatory stimulus provided by a steroid acrasin (*Δ*2-stigmasten-3β-ol) and also by the sex steroids estradiol, progesterone and testosterone in *Amoeba* sp. Similarly, Charney and ... *Euglena gracilis*, and testosterone and estrone in *Trichomonas* spp.

The histochemical demonstration of 11β-HSDH in *S. conoides* indicates its potential to synthesize cortico-steroids. This steroid dehydrogenase is known to bring about the conversions of cortisol to cortisone and 11β-androgens and 11β-estrogens to 11β-keto derivatives.

Demonstration of NADH diaphorase has been carried out, as it is a prerequisite for this histochemical procedure, since electron transfer from hydroxysteroids to the chromogenic hydrogen acceptor, NBT, is not catalyzed without the mediation of this enzyme.
G-6-PDH is an energy metabolising enzyme known to generate NADPH that is needed for the hydroxylation of steroids during steroidogenesis (McKerns, 1969). The occurrence of G-6-PDH along with 3β, 17β, 11β, hydroxysteroid dehydrogenases in the trophozoites, gamonts and zygotes of *S. conoides* provides a supporting evidence of steroidogenesis in this gregarine.
SUMMARY

1. Neutral lipids and cholesterol, the precursors of steroids, have been cytochemically demonstrated in the trophozoites, gametes and zygotes of the gregarine S. conoides. However, the lipids and cholesterol are not found in the sporozoites.

2. The presence of 3β-hydroxysteroid dehydrogenase (Δ⁵-3β-HSDH), 17β-hydroxysteroid dehydrogenase (17β-HSDH) and 11β-hydroxysteroid dehydrogenase (11β-HSDH) is cytochemically demonstrated in the trophozoites and zygotes of S. conoides. The activities of these enzymes were almost nil in the gametes and sporozoites of this species.

3. Based on our findings, it is inferred in the present work that the gregarine S. conoides has the potential to synthesize steroids. This ability is observed only in the trophic and zygote stages. The gametes, though contain the lipids and cholesterol, lack the necessary enzymes. The sporozoites neither contain the needed precursors nor the enzymes.
Table-8: 3β-HSDH, 17β-HSDH, 11β-HSDH and G-6-PDH activities in different stages of *Stylocephalus conoides* Dev.

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<th>Enzymes and substrates</th>
<th>Trophozoites</th>
<th>Gamonts</th>
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<th>Gametes</th>
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<td>Testosterone/Estradiol</td>
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<td>11β-OH-androstenedione</td>
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