CHAPTER II.

CYTOCHEMICAL STUDIES ON CERTAIN STEROID DEHYDROGENASES INVOLVED IN STEROID BIOSYNTHESIS /METABOLISM IN THE CILIATES N. cordiformis AND O. ranarum AND IN THE FLAGELLATES P. indica, S. flagellata AND H. ovalis.
INTRODUCTION

Steroids and sterols are cyclic alcohols containing cyclopentanophenanthrene rings. As they are saponified by NAOH, they form unsaponified portion of the tissue lipids. They have wide biological activities (Ghosh, 1984).

Biosynthesis of steroids and their in vitro conversion have been studied recently in many invertebrate groups, viz., echinoderms, molluscs, crustaceans, insects and nematodes (Brigs, 1972; De long camp, 1974; Sandor, 1975 a review; and Goudar et al., 1977). Likewise, steroids and sterols have also been reported in a few protozoans (Sebeck and Michaels, 1957; Conner et al., 1966; and reviews: Wagendonk, 1955; Wright, 1964; Charney and Herzog, 1967; Dewey, 1967 and Shorb, 1967; Amoji, 1975; Desai and Nadkarni, 1987; Yuki, and Csaba, 1981; Hooi, 1988; Yoshikawa, 1988; Fulop, 1988).

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 characterised by the presence of smooth endoplasmic reticulum and sperical mitochondria with tubular cristae (Christensen and Gillim, 1969). The presence of $\Delta^5$-3β-hydroxy steroid dehydrogenase ($\Delta^5$-3β-HSDH) one of the key enzymes involved in steroid biosynthesis, has been histochemically demonstrated in all the steroidogenic tissues of vertebrates (Samuels et al., 1951, von Oordt, 1963, Dorfman and Unger, 1965, Baillie et al., 1966 and Rubin et al., 1969). $\Delta^5$-3β-HSDH activity has been demonstrated in a few species of invertebrates v.i.z in a variety of tissues of the oyster Crassostrea gigas (Mori et al., 1964), in the male phase of ovo-testis Vaginulus tempitonti (Gouder et al., 1977), in the spermatids and spermatozoa of two heteropteran insects Graphosoma italicum and Eurydema ventralis (Trandabura and Tasca, 1976), in the gonads of the lepidoptera Philosoma ricini, Antitheraea mylitta and Bombyx mori (Hurkadli, 1982), in the silk worm ovaries
(Ohinishi et al., 1985), in the gonads of B. mori (Ogisho et al., 1986) and in Schistosoma sp. (Briggs, 1972).

Following is some useful information on steroidogenic potentials and steroid metabolism in protozoan species.

Sebeck and Michaels (1957) have biochemically demonstrated in vitro conversion of steroids in Trichomonas foetus, Trichomonas gallinae and Euglena gracilis which is similar to the steroid conversion found in mammalian tissues and various micro-organisms. 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. Britt and Bloch (1961) recovered the tritiated lophenol, totally unchanged from the culture medium of Tetrahymena corlissi. On the other hand Wright (1964) has shown that Enta-
**moebia coli** has the ability to incorporate radioactive stigmasterolen which suggests its ability of sterol biosynthesis. Further, Wright (1964) has observed in *Amoeba* sp. the aggregatory stimulus provided by a steroid acrasin and also by the sex steroids, oestradiol, progesterone and testosterone. Conner et al., (1966) have shown that the ciliate *Tetrahymena pyriformis* has the ability to convert cholesterol into cholesta-5, 7, 22 triene, and Csaba 3β-01. Fulop (1988) observed the effect of hormonal imprinting on tritiated steroid incorporation of *Tetrahymena pyriformis*. Inazu et al., (1994) showed biochemically the occurrence of 20α-hydroxy steroid dehydrogenase from *T. pyriformis*. Yoshi and Yukio kawa/ (1988) showed the distribution of digitonin sterol complexes in *Pneumocystis carinii*. Among sporozoa the presence of cholesterol has been reported in the gregarine *Stylocephalus mesomorphic* (Amoji, 1975), the presence of lipids, cholesterol and steroid dehydrogenases has been reported in the gregarine *Stylocephalus conoides* not only
in trophozoites but also in reproductive stages (Desai, 1980; and Desai and Nadkarni, 1987). The presence of steroid dehydrogenases and G-6-PDH has been reported in trophozoites of different gregarine species, v.i.z. Gregarina cuneata, Hirmocystis speculitermis, H. incola and Steinina terminis by Hooli (1988).

A cursory survey of the literature reveals that there is a need to carry out further work to gather some more information on steroidogenic potential of some more protozoan species, before corroborating either of the two above mentioned contradictory views on their steroidogenic capability. Therefore an attempt is made in the present study to carry out the histochemical identification of a) lipids, b) cholesterol and c) a few key steroidogenic enzymes like $\Delta^5$-3$\beta$-hydroxysteroid dehydrogenase ($\Delta^5$-3$\beta$-HSDH), 17$\beta$-hydroxysteroid dehydrogenase (17$\beta$-HSDH), 11$\beta$-hydroxysteroid dehydrogenase (11$\beta$-HSDH), in the parasitic ciliates *Nyctotherus cordiformis*
mis and Opalina ranarum from Rana tigrina and the parasitic flagellates Psuedotrichonympha indica, Spirotrichonympha flagellata and Holomastigotoides ovalis from the termite Coptotermes sp. The presence of rich amounts of lipids, cholesterol and the above mentioned dehydrogenases in an organism has been taken as an evidence of its steroidogenic potential.
MATERIALS AND METHODS

The trophozoites of *N. cordiformis* and *O. ranarum* from host frog *Rana tigerina* and *P. indica*. *S. flagellata* and *H. ovalis* from host termite *Coptotermes* sp. were procured as described in Chapter I.

A) DEMONSTRATION OF LIPIDS:

Trophozoites of ciliates and flagellates under study were examined for the presence of general lipids, neutral lipids and phospholipids by following methods: (1) Sudan Black B method, (2) Oil Red O method and (3) Nile Blue Sulphate method.

(1) Sudan Black B (SBB) Method (Barka and Anderson, 1963); Preparation of SBB solution; The staining solution 70% ethanol saturated with Sudan Black B at 4°C and filtered at same temperature.

The above said specimens were rinsed in 70% alcohol and stained with above mentioned Sudan
black B solution for 15 minutes. Then they were rinsed in 70% ethanol, washed in tap water, rinsed in distilled water and finally mounted in glycerine. Trophozoites of *N. cordiformis* treated with acetone and subsequently stained as above served as controls.

(2) Oil Red O (ORO) Method: (Barka and Anderson, 1963) Preparation of ORO solution; 0.5% Oil Red O solution was prepared in 98% isopropanol.

The fixed specimens were washed in water. They were stained in freshly prepared above mentioned ORO solution for about 30 minutes; then they were briefly differentiated in 60% isopropanol, washed in water and mounted in glycerine.

(3) Nile Blue Sulphate (NBS) Method; (Barka and Anderson, 1963); Preparation of NBS solution; 25 ml of 0.5% sulphuric acid added to 250 ml of saturated aqueous solution of Nile blue sulphate. This solution was boiled for two hours.
The specimens were fixed in calcium-formol for 10 minutes. They were stained at 60°C for 90 minutes with the above mentioned staining solution. Then they were rinsed in distilled water, placed in acetone preheated to 50°C, transferred to 5% acetic acid for 30 minutes and rinsed in distilled water. They were then washed for 3 minutes with 0.5% hydrochloric acid, rinsed in distilled water and finally mounted in glycerine.

B) DEMONSTRATION OF CHOLESTEROL AND ITS ESTERS;

Trophozoites of ciliates and flagellates under study were examined for the presence of cholesterol and its esters by adopting the methods (1) Schultz method and (2) Okamato method.

1) 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 7 days at 37°C with 2.5% ferric ammonium sulphate prepared in 0.2 M acetate buffer (pH 3.0). They were washed in acetate buffer 3 changes of 1 hour each and then with distilled water before they were treated for 10 minutes with 5.0% formalin. The specimens were treated with a drop of a mixture of conc. H$_2$SO$_4$ and acetic acids and were covered with cover slips. The preparations were examined under a compound microscope for the development of greenish blue colour.

2) Okamato method (after Udea; cited in Pearse, 1961):

Trophozoites of ciliates and flagellates under study mounted on slides were fixed in 4% formalin for 15 minutes and were thoroughly washed in distilled water. The excess water was removed by blotting paper. The specimens then were treated with 2-3 drops of freshly prepared sulphuric iodine (conc. H$_2$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.

After covering with coverslips the preparations were examined under the microscope for the development of bluish green colour.

C) DEMONSTRATION OF STEROID DEHYDROGENASES:

The trophozoites of ciliates and flagellates were rinsed in cold acetone (4°C), and were incubated in appropriate media under aerobic conditions for 30 minutes. They were then fixed in neutral formalin, washed in water and mounted in glycerine.

For the demonstration of hydroxy steroid dehydrogenases, Wattenberg's method (1958) modified by Lewy et al.,(1959) and subsequently modified by Baillie et al.,(1966) was adopted.

The incubation medium consisted of the steroid substrate 1 mg/ml. dissolved in Dimethy forma-
mide (DMF), the co-enzyme $\beta$-NAD 1.5 mg/ml., the hydrogen acceptor NBT (Nitro Blue Tetrazolium) salt 1 mg/ml. and 0.2 M 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) $\Delta^5$-3$\beta$-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$\beta$-oestradiol
   
   (3, 17$\beta$-dihydroxy-1, 3, 5(10)-estratriene).

2. Testosterone
   
   (17$\beta$-hydroxyandrost-4-en-3-one).

c) 11$\beta$-HSDH activity:

(11$\beta$-hydroxyandrost-4-en-3,17-dione).

Trophozoites of *N. cordiformis* were incubated in a substrate-free medium and served as controls.
The intensities of the above mentioned substrates and enzyme activities have been symbolically presented in a tabular form. The maximum and the minimum activities are shown as (+++++) and (+) along with other gradations in between. The results have been given in a tabular form (Table-3).

The results and conclusions have been supported by microphotographs.
A) LIPIDS:

The trophozoites of *N. cordiformis* and *O. ranarum* showed a rich distribution of Sudan black B+ (lipid) droplets (Figs. 93 & 94). The flagellate *P. indica* did not show any Sudan black B+ve droplets (Fig. 95). The trophozoites of *N. cordiformis* treated with acetone and then stained with Sudan black B did not show any SBB+ve droplets (Fig. 96). These served as controls. The trophozoites of *N. cordiformis* and *O. ranarum*; *P. indica* and *S. flagellata* showed faintly red coloured droplets (neutral lipid droplets) and could not be photographed. The trophozoites of *N. cordiformis* and *O. ranarum* showed blue droplets (phospholipids) in endoplasm with NBS (Figs. 97 & 98). The flagellate *H. ovalis* showed moderate distribution of these blue droplets (Fig. 99).
B) CHOLESTEROL:

The trophozoites of _N. cordiformis_ and _O. ranarum_ examined for Schultz reaction showed a greenish blue colour but it was a very faint and remained for only 2-3 minutes and hence could not be photographed. The flagellates _P. indica_ and _S. flagellata_ showed still more faint greenish blue colour for which reason these also could not be photographed.

The afore-said ciliates and flagellates showed very faint bluish green colour with Okamato reaction also, which lasted for a few minutes for which reason they could not be photographed.

C) STEROID DEHYDROGENASES:

1. \(\Delta^5-3\beta\)-HSDH:

The trophozoites of _N. cordiformis_ and _O. ranarum_ with both the substrates (Pregnenolone and DHEA) showed a dense distribution of blue formazan granules in the endoplasm thereby exhibiting an
intense enzyme activity (Figs.100-103). The flagellates *P. indica* and *S. flagellata* showed a sparse distribution of blue formazan granules thereby exhibiting a minimum enzyme activity, while *H. ovalis* did not show any granules exhibiting absence of enzyme activity (Figs.104-109). The trophozoites of *N. cordiformis* incubated in the substrate-free medium showed traces of activity and served as controls (Fig.110).

2. 17β-HSDH:

The trophozoites of *N. cordiformis* and *O. ranarum* with both the substrates (17β-Oestradiol and testosterone) showed a dense distribution of bluish formazan granules in the endoplasm thereby indicating an intense enzyme activity (Figs.111-114). Among the flagellates *P. indica* did not show any blue formazan granules thereby exhibiting the absence of enzyme activity, *S. flagellata* showed very few blue formazan granules thereby indicating traces of enzyme activity, while *H. ovalis* did not show any granules indicating
absence of enzyme activity (Figs.115-119). The trophozoites of *N. cordiformis* incubated in substrate-free medium showed traces of enzyme activity and served as controls (Fig.120).

3. 11β-HSDH:

The trophozoites of *N. cordiformis* and *O. ranarum* incubated in substrate medium showed a dense distribution of blue formazan granules in the endoplasm thereby indicating an intense enzyme activity (Figs.121&122). Among the flagellates *P. indica* showed sparse distribution of blue formazan granules thereby exhibiting the minimum enzyme activity, while *H. ovalis* showed no granules exhibiting no activity of the enzyme (Figs.123&124). The trophozoites of *N. cordiformis* incubated in the substrate-free medium showed traces of enzyme activity and served as controls (Fig.125).

D AND E) NADH-DIAPHORASE AND G-6-PDH:

The results of these enzyme activities in the ciliates and flagellates under study have been described in chapter-1 in the present work.
DISCUSSION

Steroids and sterols are saponified by NaOH, they form unsaponified portion of the tissue lipids. They have wide range of biological activities (Ghosh, 1984).

Barrington (1968) states that steroid biosynthesis is not an exclusive prerogative of vertebrates, since steroid biosynthesis is widespread throughout the animal kingdom. The occurrence of steroids and steroid dehydrogenases has been reviewed by Sandor et al. (1975). The occurrence of steroids and steroid in different species of protozoa free-living as well as parasitic has been reviewed by Wagendonk (1955), Wright (1964), Shorb (1964 and 1967), Dewey (1967), Charney and Herzog (1967) and von Brand (1973).

The sterols and steroids extracted from flagellates Euglena sp. and Trichomonas sp. are cholesterol, stigmasterol, β-stigmasterol, glucocorticoids, testosterone and progesterone. Some
representative species from Rhizopoda, Mastigophora and Ciliata have been studied to assess their steroidogenic potential by earlier workers (Sebeck and Michaels 1975; Wright 1964; and Conner et al., 1966). The protozoan species studied to assess their steroidogenic potential are Entamoeba coli, Amoeba sp. and Paramecium aurelia. The sterols and steroids extracted from these organisms are cholesterol, stigmasterol, β-stigmasterol, glucocorticoides, testosterone and progesterone. The symbiotic organisms of the insects Blatella germanica and Ambrossa beetles may synthesize cholesterol, the precursor of steroids (Clark and Bloch, 1959, and Norris et al., 1969). Sporozoan representatives had not been examined till recently. Only recently the presence of cholesterol has been observed in two gregarine species Stylocephalus mesomorphi (Amoji, 1975), Stylocephalus cornoides (Desai, 1980; and Desai and Nadkarni, 1987). In the latter species besides cholesterol the presence of certain key steroid dehydrogenases
has also been demonstrated by cytochemical methods. Further Hooli (1988) observed these key steroid dehydrogenases in four more species of gregarines, *Gregarina cuneata*, *Hirmocystis speculiformis*, *H. incola* and *Steinina termitis*.

Views on steroid biosynthetic potentials of protozoa are not uniform. Sebeck and Michaels (1957) have demonstrated the in vitro conversion of steroids in *Trichomonas vaginalis* and *Euglena gracilis* which is similar to the steroid conversion found in mammalian tissues and have shown that they contain steroid dehydrogenases which specifically convert C18 and C19 steroids. Charney and Herzog (1967) have studied biosynthesis of testosterone and oestrone in *Trichomonas* sp. and testesterone in *Euglena gracilis*. Wright (1964) has shown that *Entamoeba coli* has the ability to incorporate radioactive stigmasterol which suggests its ability of sterol biosynthesis. Further he has observed in *Amoeba* sp. the aggregatory stimulus provided by a steroid acrasin (22-stigma...
stenol-3β-01) and also by the sex steroids oestradiol, progesterone and testosterone. Conner et al., (1966) have shown that the ciliate *Tetrahymena pyriformis* has the ability to convert cholesterol into cholesta-5,7,22 triene 3β-01.

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 species of *Paramecium* and *Tetrahymena* and parasitic trichomonads are known to depend upon the exogenous and Csaba supply of steroids (Hutner, 1964). Kohidai (1990) stated that *Tetrahymena* sp. does not contain steroid receptors which however develop in it after steroid treatment. Gutteridge and Coombs (1977) opine that most of the parasitic protozoa cannot synthesize steroids and as such they have to procure these substances from the ambient and Csaba medium. Fulop (1988) stated that steroid pre-exposed *Tetrahymena pyriformis* cells incorporate a greater amount of steroids at the second expo-
sure owing presumably to induction of steroid receptors at the first interaction. Inazu et al. (1994) showed biochemically 20α-hydroxy steroid dehydrogenase from *T. pyriformis*. Yoshikawa et al. (1988) investigated the distribution of digitonin-sterol complexes in *Pneumocystis carinii* which may decrease with the development of organism from the trophozoite to cyst stage.

Amoji (1975) has reported the presence of free fatty acids, cholesterol and NADH diaphorase in the trophozoites of *Stylocephalus mesomorphii*. Desai (1980) and Desai and Nadkarni (1987) observed a rich amount of neutral lipids, cholesterol in trophozoites of *S. conoides* in addition certain key enzymes involved in the biosynthesis of steroid v.i.z. 3β-HSDH, 17β-HSDH and 11β-HSDH in *S. conoides*. Hooli (1988) demonstrated the activities of the enzymes 3β-HSDH and 17β-HSDH in four more gregarine species *Gregarina cuneata*, *Hirmocystis speculitermis*, *H. incola* and *Steinina termitis*. 
In the present work lipids, cholesterol, and the activities of the enzymes 3β-HSDH, 17β-HSDH and 11β-HSDH have been demonstrated in the ciliates *N. cordiformis* and *O. ranarum*. The quantity of lipids and cholesterol was less in *P. indica* and still less in *S. flagellata* and *H. ovalis*. Similarly, afore-said steroid dehydrogenases activity was minimum in *P. indica* and it was traces in *S. flagellata* and totally absent in *H. ovalis*. The results suggest that the ciliates *N. cordiformis* and *O. ranarum* possess the steroidogenic potential. The flagellates *P. indica*, *S. flagellata* and *H. ovalis* do not possess it and hence are host dependent. This observation is a suggestion that needs biochemical studies for confirmation.

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 characterised by the presence of smooth endoplasmic reticulum and spherical mitochondria with tubular cristae (Chir-
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 gigantea (Hildbrand, 1976). These structural peculiarities suggest that the protozoans may have steroidogenic potential. However, this view is subject to further confirmation by biochemical studies.

It is well known that the biosynthesis of hormonally active steroids in the steroid-producing tissues involves the conversion of Δ⁵-3β-hydroxysteroid to Δ⁴-ketosteroids and the enzyme system carrying out this reaction is shown to be Δ⁵-3β-hydroxysteroid dehydrogenase (Δ⁵-3β-HSDH). Its presence has been histochemically demonstrated in all the steroidogenic tissues of vertebrates (Samuels et al., 1951, von Oordt, 1963, Dorfman and Unger, 1965, Baillie et al., 1966 and Rubin et al., 1969). "The cytochemical demonstra-
tion of $\Delta^-3\beta$-HSDH activity in cells permits one to infer that they actively participate in the biosynthesis / metabolism of steroids" (van Oordt, 1963). The histochemical demonstration of $\Delta^-3\beta$-HSDH activity is generally considered as an evidence albeit indirect of the steroidogenic / steroid metabolic potential of any organism or tissue under consideration (Lehoux and Sandor, 1970). In the vertebrate biosynthetic scheme $\Delta^-3\beta$-HSDH and $\Delta^-\Delta^+$-isomerase effect the transformation of pregnenolone into a host of biologically active C21, C19 and C18 compounds (Lehoux and Sandor, 1970). $\Delta^-3\beta$-HSDH catalyses the conversion of pregnenolone to progesterone, 17-OH pregnenolone to 17-OH-progesterone and dehydroepiandrosterone (DHEA) to androstenedione (Baillie et al., 1966). $\Delta^-3\beta$-HSDH activity has been demonstrated in a few species of invertebrates v.i.z in a variety of tissues of the oyster *Crassostrea gigas* (Mori et al., 1964), the male phase of oovotestis of *Vaginulus templitoni* (Goudar et al., 1977).
in the spermatids and spermatozoa of two heteropteran insects Graphosoma italicum and Eurydema ventralis (Trandabura and Tasca, 1976), in the gonads of the lepidopteran Philosoma ricini and Antitheraea mylitta and Bombyx mori (Hurkadli, 1982) and in schistosomes (Brigs, 1972). Recently the presence of $\Delta^5$-3B-HSDH has been shown by cytochemical methods in the gregarine S. conoides (Desai 1980; Desai and Nadkarni, 1987), G. cuneata, H. speculitermis, H. incola and S. termitis (Hooi, 1988). The presence of enzyme with DHEA as the substrate used in the medium in the present work suggests that N. cordiformis, O. ranarum have the enzyme necessary to convert DHEA into androstenedione. The flagellates P. indica, S. flagellata and H. ovalis do not possess the enzyme.

17B-HSDH is an essential enzyme involved in the biosynthesis of sex steroids, the androgens and oestrogens. This enzyme catalyses the interconversions of testosterone to androstenedione and vice versa and 17B-oestradiol to oestrone and
vice versa in the gonads of vertebrates (Baillie et al., 1966 and Rubin et al., 1969). The occurrence of 17β-HSDH has been histochemically demonstrated in the spermatids and spermatozoa of the heteropteran insects G. italicum and E. ventralis (Trandabura and Tasca, 1976), and in the non-germinal cells of the gonads of lepidopteran P. ricini, A. mylitta and B. mori (Hurkadli, 1982).

Based on the biochemical studies Lehoux and Sandor (1970) have inferred that the gonads of insects Gryllus domesticus have two substrate specific 17β-hydroxysteroid dehydrogenases, i.e. testosterone 17β-hydroxysteroid dehydrogenases and oestradiol-17β-hydroxysteroid dehydrogenases and that the testosterone dehydrogenase is different from the oestradiol dehydrogenase. When the gonadal homogenate was incubated in the presence of both testosterone and 17β-oestradiol the yield of the 17β-ketosteroid produced were the same as that in experiments, where only one of the substrate was incubated. From this they have
inferred that there does not seem to be any competition for the active sites between the androgenic and oestrogenic precursors. Further the significant difference in-vitro conversion of testosterone (2%) and 17β-oestradiol(5%) has lead to the inference that the gonads of the mussel *Mytilus edulis* have two 17β-HSDH enzyme entities (De Long Camp, 1974). The in vitro conversion studies on the sperm preparations of oyster (Hathway, 1968) and of the gonads of the *Mytilus edulis* (De Long Camp, 1974) and *Sepia officinalis* (Carveau and Drozdovsky, 1974 and 1977) suggest the occurrence of 17β-HSDH in mollusc. The occurrence of 17β-HSDH has been histochemically demonstrated in vitro on the sperm preparations of urchins and the ovaries of *Asterias rubens* (Hathway, 1965), in the kidney and digestive tract of *Crassostrea gigas* (Mori et al., 1965) and in the male phase ovo-testis of *V. templitonii* (Goudier et al., 1977). Recently 17β-HSDH activity has been shown by cytochemical methods in the gregarine *S. conoides* (Desai, 1980; and Desai...
and Nadkarni, 1987), G. cuneata H. speculitermis, H. incola and S. termitis (Hooli, 1988). Our histo-
chemical demostration of 17β-HSDH in N. cordiform-
mis and O. ranarum using both substrates, testosterone and 17β-oestradiol suggest the presence of both steriospecific 17β-HSDH in these organisms. The presence of 17β-HSDH in N. cordiform-
mis, O. ranarum suggests that these organisms have the potential to synthesize the sex steroids. While P. indica S. flagellata and H. ovalis lack the enzyme and suggesting these are not having the potential to synthesize the sex steroids.

De Clerk et al., (1983 and 1984) have identified progesterone, testosterone and several related androgens in the larval haemolymph of Sarcophaga bullata. Ohinishi et al., (1985) have identified 17β-oestradiol from the silk worm ovaries. Ogiso et al., (1986) have observed different metabolic activities in the gonads of B. mori i.e. ovaries of B. mori converted oestrone to 17β-oestradiol and vice versa but testosterone
was not appreciably changed. Further the testes converted the testosterone to several metabolites but oestradiol was not converted like wise. Gottfried and Dorfman (1970a, 1970b & 1970c) have shown in the mollusc Arilimax californicus that dehydroepiandrosterone and 11-ketosterone can block inhibitory activity of the right optic tentacle or spermatogenesis. Mori et al., (1965) have shown that the treatment with 17β-oestradiol accelerates the sexual maturation in the female C. gigas and they have also shown the sex-reversal from male to female in this species after 17β-oestradiol administration. These studies indicate some role of steroid hormones in the regulation of sexual reproduction in invertebrates.

11β-HSDH has been demonstrated in S. conoides (Desai, 1980; and Desai and Naidkarni 1987). In the present work we could demonstrate 11β-HSDH in N. cordiformis and O. ranarum but not in P. indica.
and *H. ovalis*. This indicates the ciliates have potential to synthesize corticosteroids, while the flagellates do not have that potential. This steroid dehydrogenase is known to bring about the conversions of cortisol to cortisone and 11β-androgens and 11β-oestrogens to 11β-keto derivatives.

NADH-diaphorase is a prerequisite for the electron transfer from hydroxy steroids to the chromogenic hydrogen acceptor NBT, which is not catalysed without the mediation of this enzyme. NADH-diaphorase has been demonstrated in *S. mesomorphi* (Amoji, 1975), in *S. conoides* (Desai, 1980; and Desai and Nadakarni, 1987) and in *Stylocephalus* sp. (Vinodini, 1991). We have demonstrated this enzyme in *N. cordiformis*, *O. ranarum* and *P. indica* but not in *S. flagellata*.

Glucose-6-phosphate dehydrogenase (G-6-PDH) is involved in generating the reduced nicotinamide adenine dinucleotide phosphate (NADPH) which is
required for hydroxylation of steroids during active steroidogenesis (White et al., 1959; Savard et al., 1963; McKerns, 1968 and Weist and Kidwell, 1969). Further, G-6-PDH is implicated along with \( \Delta^5 - 3\beta\)-HSDH in enzymatic transformation related to steroidogenesis in vertebrates (Weist and Kidwell, 1969).

G-6-PDH activity has been demonstrated in the ciliate *Balantidium coli* (Sharma and Bourne, 1964b), in *Nyctotherus georgei*, *Opalina ranarum* and in the gregarines *Stenophora conjugata* and *S. mesomorphi* (Amoji, 1975), in *S. conoides* (Desai, 1980; and Desai and Nadkarni, 1987), in *G. cuneata*, *H. speculitermis*, *S. termitis* and *H. incola* (Hooli, 1988), in *Stylocephalus* sp. (Vinodini, 1991), and in coccidians *Eimeria stiedae* (Frandsen, 1970). Further G-6-PDH activity has been biochemically observed in *Eimeria stiedae* (Frandsen, 1975 a, b and 1976 and Frandsen and Ennis, 1974). In the present work this enzyme activity has been demonstrated in *N. cordiformis*, *O. ranarum*,

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P. indica and S. flagellata, N. cordiformis and O. ranarum possess all the enzymes i.e. 3β-HSDH, 17β-HSDH, 11β-HSDH, NADH-diaphorase and G-6-PDH suggesting their steroidogenic potential. While P. indica lacks the enzymes 3β-HSDH, 17β-HSDH and 11β-HSDH but possesses NADH-diaphorase and G-6-PDH which might be having some other role other than steroidogenic.

It may be assumed that the moulting hormones (steroid) of the termite host during the frequent moults (once in a month) serve as a source of steroid for flagellate parasites. The presence of the enzymes of steroid biosynthesis in Psuedotrichorympha indica may also reflect its phylogenetic connection with the ancestral psuedotrichonymphs which parasitised ancient Cryptocercus roaches with their infrequent moults (once a year) (Bobyleva and Podlipaeva, 1996 .... personal communication).
In young and adult frogs, there will be a secretion of both the growth and sex hormones. The ciliate parasites show the trophic growth and also reproduce asexually. In the tad-pole stage there will be the secretion of growth hormone prolactin and metamorphosis hormone thyroxine. But the ciliate parasites reproduce sexually indicating the ciliate parasites synthesize the required sex hormones during their stay in this host.

In termites the worker caste itself is a sterile group. In the presence of the growth hormone, ecdysone, of the host the flagellates reproduce only asexually.

The flagellates of the genus Trichonympha from two host groups, v.i.z. Cryptocercus and lower termites underwent different evolutionary changes at about the same period of time. The evolution of trichonymphs from Cryptocercus was a divergent speciation. At the same time in termites Trichonympha species showed resistance and stability, which seems to be determined by transi-
tion of the flagellates to an exclusively agamic mode of reproduction (Bobyleva, 1975). We propose to put-forth our views in this connection. In most of the parasites the reproductive events are well regulated and monitored by the hormones of their hosts. In the roach *Cryptocercus* there is a periodical surge of hormones monitoring the sexual activities of not only the roach but also of the *Trichonympha* species inhabiting the gut of this roach. It would be interesting to examine these parasites to ascertain by histochemical/biochemical methods if the latter themselves have the potential for synthesizing the sex hormones or they are also host-dependent for this purpose. In case of *Trichonympha* and members of other genera of flagellates of termites the situation is different. Their hosts, v.i.z. the termite workers being sterile forms can not secrete hormones periodically once their final moulting is over. At the same time their intestinal flagellate species also lack the enzyme machinery needed for the
biosynthesis of sex steroids as our histochemical studies have revealed. For this reason they might have skipped the gametogenic phase in their life-cycle. Further studies on the parasitic protozoan fauna of these two host groups are needed to attest the validity of our views.
SUMMARY

1. Neutral lipids and cholesterol, the precursors of steroids have been cytochemically demonstrated in the trophozoites of afore-said ciliates and flagellates. However, the lipids and cholesterol were less in the flagellates.

2. The occurrence of $\Delta^5$ - 3β - HSDH in both the ciliates indicates their potential to synthesize / metabolise the steroids. The activity of this enzyme was almost nil in the flagellates.

3. The occurrence of 17β-HSDH with both substrates i.e. testosterone and 17β-oestradiol, suggest the potential of these ciliates to synthesize / metabolise the sex steroids in a manner similar to higher invertebrate and vertebrate gonads. The activity of this enzyme was almost nil in the flagellates.

4. The occurrence of 11β-HSDH indicates the conversion of cortisol to cortisone and 11β-androgens and 11β-oestrogens to 11β-keto
derivatives. Again this enzyme activity was almost nil in the flagellates.

5. The occurrence of G-6-PDH activity in the ciliates provides an additional evidence, albeit indirect, of the steroidogenic potentiality of these organisms, as it is known to generate the NADPH required for the hydroxylations of steroids during steroidogenesis. Absence of steroid dehydrogenases in the flagellates suggests that these parasites are host-dependent for their steroid requirement.
<table>
<thead>
<tr>
<th>A. Substrates</th>
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<tr>
<td><strong>I. Lipids</strong></td>
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<tr>
<td>SBS</td>
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<td>ORO</td>
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<td>NBS</td>
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<td><strong>II. Cholesterol</strong></td>
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<tr>
<td>Schultz reaction</td>
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<td>Okamato reaction</td>
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<th>B. Enzymes</th>
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<tr>
<td><strong>I. Steroid dehydrogenases</strong></td>
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<tr>
<td>1. ( \Delta^5 ) - 3β - HSDH</td>
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<tr>
<td>Pregnenolone/DHEA</td>
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<tr>
<td>Substrates and N.cordiformis O.ranarum P.indica S.flagellata H.ovalis Control</td>
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<td>---------------------------------------------------------------</td>
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<tr>
<td><strong>Enzymes.</strong></td>
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<td>2.17β- HSDH</td>
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<td>Testosterone/</td>
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<td>3.11β- HSDH</td>
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**+++ = maximum reaction; +++ = fairly intense reaction; ++ = moderate reaction**

**+ = minimum reaction; +/- = traces of reaction; - = absence of reaction**

**= not examined.**
Explanation to Figures

Figs. 93 to 96 show Sudan Black B (SBB) reaction.

Fig. 93. *Nyctotherus cordiformis*, x 1000. Note the SBB positive lipid droplets.

Fig. 94. *Opalina ranarum*, x 1000. Note the SBB positive lipid droplets.

Fig. 95. *Psuedotrichonympha indica*, x 1000. Note the absence of SBB positive lipid droplets.

Fig. 96. *N. cordiformis*, x 1000. Note the absence of SBB positive lipid droplets. A control preparation.

Figs. 97 to 99 show Nitro Blue Sulphate (NBS) reaction.

Fig. 97. *N. cordiformis*, x 1000. Note the phospholipids concentrated in the nucleus and uniformly distributed in the cytoplasm also.

Fig. 98. *O. ranarum*, x 1000. Note the phospholipids concentrated in the nuclei and uniformly distributed in the cytoplasm also.