CHAPTER 3

HISTOCHEMICAL STUDIES ON STEROIDOGENIC CELLULAR SITES

IN THE OVARIES OF CALOTES VERSICOLOR, HEMIDACTYLIUS

FLAVIVIRIDIS AND CHAMASOLON CALCARATUS AND CYCLIC

CHANGES IN THE ACTIVITY OF STEROID CONVERTING ENZYMES

IN THE OVARY OF C. VERSICOLOR
The vertebrate ovary as the internal secretor of steroid hormones has been a subject of considerable research. During the past decade or so, the discoveries of sensitive and precise methods of investigations have made a notable contribution to the rapid development of our knowledge of steroidogenic elements in the ovaries of vertebrates (Deane and Rubin, 1963; Saillio et al., 1966; Barr, 1969; Lofts and Bern, 1972; Guraya, 1976). The reptiles in this respect are the least studied amongst the vertebrates. The biochemical separation of steroids from the reptilian ovary and their identification is limited to the reptilian species, *Lacerta sicula* (Chieffi, 1966; Iupo et al., 1967, 1968). Studies on *in vitro* conversion of steroids have been carried out on the ovary of reptiles, *Natrix nixoidon*, *Coluber constrictor*, *Thamnophis sirtalis* (Callard and Leatham, 1965). *L. sicula* (Callard et al., 1968) and *Dipsosaurus dorsalis* (Callard et al., 1972). Similar studies have been carried out on the corpora lutea of *Xantusia vigilis* (Yaron, 1972) and *Chelydra chelydra serpentina* (Klicks and Mahmoud, 1972). In these studies the ovarian homogenate or the slices are incubated in the media containing known steroid precursors and the converted products of the precursors are identified. Hence the *in vitro* conversion studies enable one to understand not only
the nature of biosynthetic pathways but also the occurrence, though indirectly, of enzyme systems catalysing these conversions. From these studies the biosynthetic pathway of steroids in the ovary of reptiles has been depicted (Ghieffi, 1966; Callard et al., 1972; Lorenzo et al., 1974). But the information gained as a result of biochemical and in vitro studies though important in many ways do not lend any clue either to the identification or location of the steroidogenic cells in the ovaries.

Certain histochemical procedures are used in localizing the steroidogenic elements in the vertebrates tissues. The presence of steroid precursors such as lipids and cholesterol can be histochemically demonstrated and the localization of these precursors is used for identifying steroidogenic cellular sites. The ovaries of *Naja triptadina*, *Bungarus coeruleus* (Guraya, 1965) and *C. versicolor* (Guraya and Verma, 1973), have been studied by means of this procedure. Histochemical demonstration of lipids in reality is insufficient to indicate steroidogenesis in tissues (Sarr, 1968). In recent years a better and more precise histochemical method has been developed originally by Wattenberg (1958) and later adapted by other workers (Levy et al., 1959; Pearson and Gross, 1959; Balloq, 1964; Baillie et al., 1966) for demonstrating steroid
converting enzymes, the hydroxysteroid dehydrogenases.

Hydroxysteroid dehydrogenase (HSDH) enzymes catalyse the oxidative conversion of hydroxysteroids to ketosteroids. In this process, the transfer of electron (H) from hydroxy-bond (OH) from a specific position of the steroid nucleus to the coenzyme NAD or NADPH is catalysed by hydroxysteroid dehydrogenases. Thus NAD or NADPH is reduced to NADH or NADPH. The electron from NADH or NADPH is further transferred to the cytochrome system of the cells by another enzyme, NADH or NADPH diaphorase. In Wattenberg’s technique, the electron transfer from hydroxysteroids transferred by the hydroxysteroid dehydrogenases to NAD or NADP is further transferred by NADH or NADPH diaphorase to Nitro Blue Tetrazolium (NBT) salt which is a chromogenic hydrogen acceptor. NBT which is a creamy white powder is thus reduced to dihydroazan that is deposited in the form of fast dark blue granules at the site of steroidogenesis. The hydroxysteroid dehydrogenases are substrate specific and hence the individual enzymes can be recognised by this method.

The specificity of the histochemical method has been ascertained by other workers (Baillie et al., 1966; Hoyer and Anderson, 1970).

Histochemical demonstration of hydroxysteroid dehydrogenases has, thus, come to be a more sensitive and
reliable tool for detecting the cellular sites of steroid biosynthesis which cannot be made out by biological experiments alone (van Groet, 1963; van Tienhoven, 1966). Further, the histochemical demonstration of hydroxysteroid dehydrogenase might give a clue to certain steps in the biosynthetic pathways of steroidogenesis.

\[ \Delta^5-3\beta\text{-HSDH} \] (Delta-5 3-beta hydroxysteroid dehydrogenase) is an important enzyme essential in the early biosynthesis of steroids. It converts \( \Delta^5-3-\beta \) hydroxysteroids to \( \Delta^4-3 \)-ketosteroids in the presence of another enzyme, \( \Delta^5-\Delta^4 \)-isomerase (which shifts the double bond between 4 and 5 to 3 and 4 position of steroid nucleus). The conversion of pregnenolone to progesterone, 17\( \alpha \)-hydroxy-pregnenolone to 17\( \alpha \)-hydroxyprogesterone and DHEA (dehydroepiandrosterone) to androstenedione are catalysed by this enzyme. Hence the presence of this enzyme is accepted as a reliable evidence of steroid hormone biosynthesis (Callard, 1972). 17\( \beta \)-HSDH is another enzyme that brings about the reversible conversion of testosterone = androstenedione and 17\( \beta \)-estradiol = estrone. Biosynthesis of sex steroids (androgens and estrogens) is inferred from the presence of this enzyme. 11\( \beta \)-HSDH is yet another enzyme of the interrenal tissue of all the vertebrates studied so far. It leads to the electron transfer from \( \text{OH} \) bond at 11\( \beta \)-position.
of the steroid nucleus of 11β-hydroxysteroids, thus 
oxidising them to 11-ketosteroids. Cortisol is oxidised 
to cortisone by this enzyme and its occurrence in stereo-
idogenic cells is taken to denote the biosynthesis of 
corticosteroids, 11β-androgens and 11β-estrogens.

\(\Delta^5\)-3β-HSDH activity has been histochemically 
demonstrated in the ovaries of \(L.\) sicula (Bottes and Delrio, 
1965), \(N.\) sipador pictiventris (Callard, 1966a,b), \(Lacerta\) 
vivipara, \(Vipera aspis\) (Morat, 1969), \(Xantusia vigilis,\) 
\(Mabuya capensis\) (Yaron, 1972) and \(Anolis carolinensis\) (Jones 
et al., 1974). This enzyme is claimed to be absent from the 
ovary of \(Hemidactylus flaviviridis\) (Prasad and Sanyal, 1966). 
There is only one report that claims the occurrence of 17β-
HSDH in the ovary of \(X.\) vigilis and \(M.\) capensis (Yaron, 1972). 
This enzyme could not be histochemically demonstrated in the 
ovary of \(L.\) sicula (Bottes and Delrio, 1965). Similarly there 
are no reports on the occurrence of 11β-HSDH in the reptili-
ian ovary, though its presence has been indirectly suggest-
ed from the in vitro study on the ovary of \(X.\) vigilis 
(Lorenzo et al., 1974). However, this enzyme has been 
histochemically demonstrated in the ovary of mouse (Saillie 
et al., 1966).

So far, identification of steroidogenic tissues is
based on the histochemical demonstration of sudanophilic lipids and HSDHs carried out only during certain phases of the ovarian cycle in reptiles and probably for this reason these reports are not in full agreement with one another and they are somewhat divergent.

The above mentioned reports suggest that there is a need to identify the steroidogenic cells of the reptilian ovary. Hence the present study was undertaken to investigate the occurrence of some steroid converting enzymes in the ovaries of reptiles. For this purpose the histochemical demonstration of $\Delta^5-3\beta$-HSDH, 17$\beta$-HSDH, 11$\beta$-HSDH, G-6-PDH, NADH diaphorase, LDH and ICOD enzyme activities in the ovary of C. versicolor has been investigated every month for a period of one year. Thus the present study is an attempt to find out the localization of these enzymes in the ovary during different phases of the reproductive cycle in order to identify the relative steroidogenic capacity of different ovarian elements in different phases of the reproductive cycle. The histochemical distribution of lipids in the ovary of C. versicolor has also been studied as an additional evidence for steroidogenesis. To have a comparative account, the above mentioned histochemical studies were also extended to the ovaries of the wall lizard, H. flaviviridis and the chameleon, Chamaeleon calcaratus.
MATERIAL AND METHODS

Live specimens of *C. versicolor*, *H. flaviviridis*, and *C. calcaratus* were sacrificed for the study within two days after they were brought into the laboratory. Monthly histochemical studies were carried out on the ovary of *C. versicolor* and the ovary of *H. flaviviridis* was studied once in three months for a year. As the live specimens of the chameleon, *C. calcaratus* were available only during the period from June to September, the ovary during that period only was investigated.

The animals were suddenly decapitated and the ovaries were removed. Pieces of ovaries mounted on block holders were quickly frozen over dry ice vapours and 14 μm sections were serially cut in 'Pearse-slice' cryostat maintained at −20°C. The frozen sections were picked up on clean cover glasses, thawed at room temperature and aerobically incubated in appropriate media at 37°C for different duration. The optimum incubation time for \( \Delta^5 \)-3β-HSDH, 17β-HSDH was one hour whereas for other enzymes, G-6-PDH, NADH diaphorase, ICDH and LDH, it was only 20 minutes. The incubation was arrested by fixing the sections in neutral 10% formalin for about 6 hours. The sections were washed in distilled water and finally mounted in glycerol gelatine.
medium. Some sections in which the lipid droplets were considerable, were either washed in cold acetone prior to incubation or in DMF (dimethyl formamide) after fixation in order to remove excessive lipid droplets and the crystal deposits.

The extent of deposition of dark blue diformazan granules was taken to indicate the intensity of enzyme activity and the latter was subjectively graded by visual assessment as follows:

1) Maximum activity = +++++
2) Minimum activity = +
3) Traces of activity = ±
4) No activity = -

The media for localizing \( \Delta^5-3\beta-HSDH \), \( 17\beta-HSDH \), \( 11\beta-HSDH \), NADH diaphorase, G-6-PDH, ICDH and LDH enzymes were prepared individually. For the histochemical demonstration of \( \Delta^5-3\beta-HSDH \), Wattenberg's (1958) method modified by Levy et al., (1959) and subsequently modified by Baillie et al., (1966) was adopted. Histochemical demonstration of G-6-PDH was carried out according to Cohen's (1959) procedure modified by Bara (1965). The LDH and ICDH activities were demonstrated according to the procedure described by Pearson (1975). Sudan Black B and Oil Red O
methods were carried out for histochemical localization of neutral lipids as given by Pearse (1972). Cholesterol and its esters were histochemically localized by Schultz technique (Pearse, 1975).

The incubation medium for the demonstration of hydroxysteroid dehydrogenases, consisted of the steroid substrate dissolved in DMF, and \( \beta \)-NAD (nicotinamide adenine dinucleotide) coenzyme and the hydrogen acceptor, NBT (Nitro Blue Tetrazolium) salt dissolved in 0.2 M phosphate buffer at 7.4 pH added together in the final concentration of the steroid, 1 mg/ml; \( \beta \)-NAD coenzyme, 1.5 mg/ml; and NBT, 1 mg/ml.

The incubation medium for the demonstration of G-6-PDH activity, contained the substrate, D-glucose-6-phosphate disodium salt (1.5 mg/ml); the coenzyme, NADP (nicotinamide adenine dinucleotide phosphate) (0.5 mg/ml and NBT (0.5 mg/ml) dissolved in 0.2 M phosphate buffer at 7.2 pH. The medium containing (0.5 mg/ml) reduced NAD (NADH) and (0.5 mg/ml) NBT dissolved in 0.2 M phosphate buffer (7.2 pH) was used for incubating section for NADH diapharase activity. Similarly, the media for IDH and LDH activity contained D-L sodium isocitrate and sodium lactate dissolved in phosphate buffer (0.9 M at 7.2 pH) in
the final concentration of 0.5 mg/ml. The coenzyme used in the media for XCDH was NADP (0.5 mg/ml) whereas for LDH, NAD (0.5 mg/ml) was used.

For controls, parallel sections were incubated in the media individually lacking either the substrate or the coenzyme. The following substrates were used for the demonstration $\Delta^5$-$3\beta$-HSDH enzyme activity:

1. Pregnenolone
   
   $\left(3\beta$-hydroxy-5-en-20-one\right)

2. 17$\alpha$-hydroxy pregnenolone
   
   $\left(3\beta$-, 17$\alpha$-dihydroxy-5-en-20-one\right)$

3. Dehydroepiandrosterone (DEA)
   
   $\left(3\beta$-hydroxyandrost-5-en-17-one\right)$

4. Etiocholanolone
   
   $\left(3\beta$-hydroxy-5-androstan-17-one\right)$

For $17\beta$-HSDH activity the following substrates were used in the incubation media:

1. 17$\beta$-estradiol
   
   $\left(3,17\beta$-dihydroxy-1,3,5 (10)-estratriene\right)$

2. Testosterone
   
   $\left(17\beta$-hydroxyandrost-4-en-3-one\right)$.
The substrate used for the demonstration of 11β-4HSDH was:

11β-hydroxyandrostenedione

(11β-hydroxyandrost-4-en-3,17-dione)

All the chemicals used in the present work were of Sigma grade obtained from the Sigma Chemical Co., U.S.A.

OBSERVATIONS

The activity of the enzymes, cholesterol and sudanophilic lipids were, in general, noticed in (1) the patches of cells in those interna of normal and atretic follicles (Fig. 3.1-3.7), (2) granulosa cells of only large preovulatory follicles and the atretic follicles of early stages (Fig. 3.1-3.13), (3) granulosa lutein cells of postovulatory follicles (Fig. 3.14-3.20), (4) interstitial gland cells of the ovarian stroma (Figs. 3.20-3.24) and the ooplasm of the oocyte of the normal follicles (Figs. 3.1-3.3).

The histochemical distribution of enzymes, lipids and cholesterol in the ovaries of C. versicolor, H. flaviviridis and C. calcaratus was quite similar. The intensity of reaction for the individual enzymes as judged by the dark blue diformazan granules varied (Table 1).
$\Delta^5$-3$\beta$-HSDH activity in the ovarian tissues was generally more than that of 17$\beta$-HSDH and 11$\beta$-HSDH enzymes. G-6-PDH, NADH diaphorase, ICDH and LDH enzymes and sudenophilic lipids in general showed maximum reaction. The intensity of $\Delta^5$-3$\beta$-HSDH activity varied depending on the $\Delta^5$-3$\beta$-hydroxysteroid substrates used in the incubation media for the demonstration of this enzyme (Table 1). The reaction with the medium containing DHSA or etiocholanolone was more than one with pregnenolone. Only traces but positive reaction could be found for 17$\beta$-HSDH and 11$\beta$-HSDH enzymes. The sections treated with cold acetone prior to incubation showed some what less diformazan granules as the fat droplets containing false localization were removed. However, in both sections treated with or without cold acetone, diformazan granules were found in the same region of the steroidogenic cells. Thus the diformazan granules in the ovarian elements indicated that the localization was not due to false or nonspecific reaction.

**Normal follicles:** The activity of enzymes was noticed mainly in patches of cells in theca interna and in the ooplasm of the normal follicles (Figs. 3.2-3.4). The activity of enzymes in the ooplasm varied with the size of the normal follicles. In normal postvitellogenic
folicles of small size (upto 4 mm diameter), the enzyme activity in the ooplasm was spread out immediately around the germ vesicle whereas it was concentrated along the periphery of the ooplasm in larger normal follicles. In the largest normal ovulatory follicles (9.0 mm diameter) the enzyme activity was found in the granulosa cells in addition to that found in oocyte and theca interna cells (Figs. 3.1-3.3).

Atretic follicles: In the follicles of early atresia, an intense activity of enzymes was noticed in the polymorphic granulosa cells and in patches of cells of theca interna (Figs. 3.5, 3.6, 3.13). The intensity of enzyme activity in the granulosa cells seemed to persist till they atrophy and are phagocytosed. However, the patches of theca interna cells showed continued activity even after follicular atresia was over and the connective tissue remnants of the atretic follicles merged with the ovarian stroma.

Corpora lutea: The luteinized granulosa cells in the postovulatory follicles of the three lizards revealed a markedly increased activity of enzymes till the luteal bodies regressed (Figs. 3.14-3.20). No enzyme activity could be discerned in the degenerated corpora lutea or
corpora albicans (Fig. 3.25). There was no appreciable activity in theca interna of the postovulatory follicles, in comparison to that of atretic and normal follicles in which the enzyme activity has been noticed.

**Ovarian stroma** : The interstitial gland cells of the ovarian stroma of the three lizards showed intense $\Delta^5-3\beta$-HSDH activity (Figs. 3.20, 3.21, 3.23 and 3.24). The intensity of $17\beta$-HSDH enzyme activity (Fig. 3.22) was considerable when compared to that in other elements of the ovary. Traces of activity of $11\beta$-HSDH was more or less as in other steroidogenic elements of the ovary. The activity of other enzymes, C-6-PDH, NADH diaphorase, ICDH and LDH in the interstitial cells of the ovarian stroma was generally high.

**Cyclic changes** : Though it was not possible to arrive at definite conclusions on the seasonal changes in the activity of enzymes in ovary of *C. versicolor*, some broad-based generalizations could be made from the monthly histochemical studies. The activity of the enzymes in the individual steroidogenic cells did not vary depending upon the phase of the reproductive cycle. During the regeneration phase (January to April) of the ovary in *C. versicolor*, the activity of the enzymes in the interstitial stromal cells of the ovary appeared very intense when compared to that of
those cells in other phases of the reproductive cycle. This was apparently due to the increase in the number of stromal cells rather than any increased enzyme activity in the individual cells. Activity of enzymes in granulosa lutein cells was observed in corpora lutea which appeared in May and persisted till December. The number of follicles undergoing follicular atresia was more during the prebreeding and postbreeding months and hence the enzyme activity in the atretic follicles apparently seemed to be more during these periods of the ovarian cycle in C. versicolor.

**DISCUSSION**

In reptiles, earlier investigators have made a few attempts to identify the ovarian steroids and the cells that synthesize them. Our knowledge on the occurrence of steroids is based on the chromatographic separation as well as in vitro steroid conversion studies carried out on the ovaries of *Coluber constrictor constrictor*, *Natrix sipedon pictiventris*, *Thamnophis airtalis airtalis* (Callard, 1966) and *Lacerta sicula* (Lupo et al., 1967, 1968). The steroids, namely, pregnanolone, 17α-hydroxyprogrenolone, DHEA, androstenedione, 17β-estradiol and estrone have been identified from the ovaries of these reptiles. It is of interest
to note that Callard (1966) and also Lupo and his colleagues
(1968) found testosterone but not estrogens in the ovarian
extracts. According to them, absence of estrogens is
either due to their active conversions or to their occur-
rence in quantities which are beyond the sensitivity level
of the experimental procedure adopted by them.

On the basis of these and other investigations,
essentially all the steps of the two known pathways of
steroidogenesis - \( \Delta^4 \) - and \( \Delta^5 \) - pathways - have been
suggested to occur in the reptilian ovary (Chieffi, 1966;
Callard, 1972; Yaron, 1972; Lorenzo et al., 1974). \( \Delta^4 \)
pathway leads from pregnenolone to progesterone and 17\( \alpha \)-
hydroxyprogesterone to androstenedione = testosterone.
\( \Delta^5 \) - pathway of steroids leads from pregnenolone to 17\( \alpha \)-
hydroxyprogrenolone to DHSA = androstenedione. A comparison
of biosynthetic pathways of steroids in the reptilian ovary
to those of other vertebrates (Nandi, 1967; Barr, 1968;
Lambert, 1970; Xavier and Oxon, 1971; Redshaw and Nicholls,
1971; Yaron, 1971; Silkes, 1972) indicates that the bio-
synthetic pathway of steroidogenesis is essentially the
same as in all other vertebrates (Callard, 1972; Yaron,
1972; Lorenzo et al., 1974).

Identification of steroidogenic cells in the ovaries
of reptiles has been studied by carrying out the histochemical
demonstration of lipids, cholesterol and hydroxysteroid dehydrogenases. Lipids and cholesterol form the precursors of steroids and the hydroxysteroid dehydrogenases catalyse certain conversions during the biosynthesis or metabolism of steroid hormones. Histochemical demonstration of sudanophilic lipids and cholesterol, both free and esterified forms, in the ovaries of Raja tripudians, Bungarus coerulescens (Guraya, 1965), C. versicolor (Varma and Guraya, 1973 a,b), H. flaviviridis (Guraya and Verma, 1976 and Anolis carolinensis (Guraya, 1976) has shown the occurrence of these steroid precursors in the interstitial gland cells and the luteinized granulosa cells of the postovulatory follicles. Thus the interstitial cells and the granulosa lutein cells have been claimed to be the steroidogenic sites in the ovaries of reptiles (Guraya, 1976). Further these studies have indicated that the atretic follicles are involved in steroid biosynthesis and that the cells of theca interna transform into interstitial gland cells of the ovarian stroma after follicular atresia. However, the results of the histochemical observation of $\Delta^5$-3β-HSDH which indicates the steroid biosynthetic potency of the ovarian elements in reptiles are not in unison with the findings based on the histochemical demonstration of sudanophilia and cholesterol. The histochemical studies
carried out so far on the ovaries of *L. aicula* (Botte Delrio, 1965), *N. aipadon pictiventris* (Callard, 1966), *Lacerta vivipara, Vipera aspis* (Morat, 1969) and *Anolis carolinensis* (Jones et al., 1974) have shown that the activity of $\Delta^5-3\beta$-HSDH enzyme occurs mainly in granulosa and to a lesser extent in the patches of cells of theca interna of normal follicles and the granulosa lutein cells of corpora lutea. Activity of this enzyme has been reported only in the granulosa cells of normal follicles in the ovaries of *Sceloporus cyanogenys* and *Dipsosaurus dorsalis* (Callard, 1972). Thus, according to histochemical observations of $\Delta^5-3\beta$-HSDH, granulosa cells and theca interna cells of normal follicles and the luteal cells of corpora lutea are the sites of steroid biosynthesis, whereas, the studies on lipids and cholesterol claim interstitial gland cells, theca interna cells of atretic follicles and granulosa lutein cells of postovulatory follicles to be the steroidogenic sites in the ovaries of reptiles.

*In vitro* conversion of C21 steroids to C19 and C18 steroids observed in the ovaries of *L. aicula* (Lupo et al., 1967, 1968) and snakes, *N. aipadon, Colubor constrictor* and *Thamnophis sirtalis* (Callard and Leatham, 1965; Callard, 1966) indirectly suggests the presence of $17\beta$-HSDH activity
involved in the biosynthesis of sex steroids in the ovary of these reptiles. NADP dependent 17β-HSD activity has been histochemically demonstrated in the granulosa cells of normal follicles in the ovary of only two species of reptiles, Xantusia vigilis and Mabuya capensis (Yaron, 1972). However, this enzyme could not be observed in the cells of theca interna. Thus the presence of 17β-HSD has led to the inference that the granulosa cells are involved in the biosynthesis of sex steroids in the presence of the coenzyme NADP but not NAD, in the ovaries of these reptiles.

11β-HSD activity is reported in the interrenal or adrenocortical cells of all the vertebrate classes (Baillie et al., 1966; Lofts and Dern, 1972) and its presence in the ovaries has been demonstrated only in mouse (Baillie et al., 1966) and fish (Nadkarni, unpublished data). As yet, there are no reports on the occurrence of 11β-HSD activity in the ovary of reptiles though the results of in vitro biosynthesis of 11β-deoxycorticosterone by the ovary of X. vigilis (Lorenzo et al., 1974) suggests, albeit indirectly, the presence of this enzyme. The thirteen-lined ground squirrel has the peculiarity of responding to adrenalectomy by forming an adrenocortical-like tissue in its ovary. The ovary of intact anoestrous
animal seems to form corticosterone as the chief metabolite of progesterone (Vinson, 1963, 1965). Corticosterone has been recovered from the incubation of human ovary with progesterone as the substrate (Richardson, 1967). Thus, the histochemical demonstration of traces but positive activity in the luteinised granulosa cells of the ovaries of the three lizards, *C. versicolor*, *H. flaviviridis* and *C. calcaratus* forms the first report on the occurrence of $11\beta$-HSDH enzyme in the ovary of reptiles. Nevertheless, the presence of this enzyme in the reptilian ovary suggests that the ovary is capable of synthesizing corticosteroids in addition to progesterone and sex steroids. $11\beta$-HSDH in adrenocortical tissues, is known to catalyse cortisol to cortisone and corticosterone to dehydrocorticosterone. However, its occurrence in the ovarian tissues might be for metabolising $11\beta$-androgens and $11\beta$-oestrogens to $11\beta$-keto sex steroids (Baillie et al., 1966).

Amongst the $\Delta^5$-$3\beta$-hydroxysteroid substrates used for the demonstration of $\Delta^5$-$3\beta$-HSDH enzyme activity, the intense reaction with DHSA might indicate its preferential utilization in vivo to the other substrates, pregnenolone and $17\alpha$-hydroxyprogrenolone. Similarly, the intense reaction observed in the sections incubated in the medium containing etiocholanolone may be due to the presence of
Reducible H at 3β- and 5β- positions of its steroid nucleus. Similar observations have been made in a number of studies made earlier on the steroidogenic cells in vertebrates (Baillie et al., 1966; Bara, 1968; Saidapur and Nadkarni, 1973; Hooli and Nadkarni, 1974; Bhujle and Nadkarni, 1975). Histochemical demonstration of G-6-PDH, NADH diaphorase, LDH and ICDH enzymes as well as sudanophilic lipids at the sites of hydroxysteroid dehydrogenases, provides an additional proof for localising the steroidogenic elements in the ovarian tissues. G-6-PDH of the monophosphate shunt, generates NADPH that is essential in hydroxylation of steroids during steroid biosynthesis. Demonstration of NADH diaphorase has been carried out as it is a necessary prerequisite for this histochemical procedure, since electron transfer from hydroxysteroids to the chromogenic hydrogen acceptor, NBT, without the mediation by this enzyme, cannot be catalysed. ICDH and LDH provide energy needed for the biosynthetic conversions of steroids.

Normal follicles: In the three lizards, occurrence of Δ^5-3β-HSDH, 17β-HSDH and 11β-HSDH enzymes in the ovaries suggests that patches of theca interna cells and to a lesser extent the oocyte and granulosa of large preovulatory follicles form the sites of steroid
biosynthesis. Amongst normal follicles, the granulosa cells of only small follicles do not show the activity of steroid converting enzymes and it is only after the normal follicles grow to certain size that the granulosa cells transform into steroidogenic cells. Thus the granulosa cells seem to develop the steroid biosynthetic activity that correlates with the size or growth of the normal follicles. Similar observation has been made on the ovary of A. corolinensis in which the granulosa cells transform into secretory cells only when the normal follicles grow to a certain size (Jones et al., 1974). In the ovary of several birds that have been histochemically investigated, the steroidogenic potency has been demonstrated to appear in the granulosa just prior to ovulation (Bhujle, 1977). In electron microscopic studies, the granulosa cells, prior to ovulation in birds, start showing agranular endoplasmic reticulum and tubular cristae in mitochondria, that is characteristic of steroidogenic cells in vertebrates (References in Lofts and Morton, 1973). Hence there seems to be a close identity in the disposition of steroidogenic sites in the ovarian normal follicles of reptiles and birds. Our results show that the granulosa cells lining the vascular theca interna and establishing communication with it, become steroidogenic. As estrogen appears to be a consistent consequence of
follicular development (Tofts and Bern, 1972), granulosa cells of large follicles might be associated with estrogen production.

There is a faint but positive reaction for the steroid converting enzymes in the oocytes of the normal follicles. The disposition of dihydroxyan granules indicative of the presence of steroid converting enzymes in the oocyte, appears well spread out around the germ vesicle and later the granules concentrate along the periphery in large follicles in which the granulosa cells become steroidogenic. So far there are no reports on the presence of hydroxysteroid dehydrogenases in the oocyte of reptiles though this feature is observed in the oocyte of fishes (Lambert, 1970) and birds (Bhuija, 1977). Guraya (1965) in his studies on the histochemical distribution of lipids in the ovaries of two snakes, has reported the occurrence of lipoproteins, phospholipids and triglycerides in the ooplasm in which these granules (which he refers as L1, L2 and L3 bodies) appear around the germ vesicle and later move towards the periphery. However, he has not attributed any steroid secretory activity to the oocyte though he noted these steroid precursors in the oocytes. The presence of lipids and steroid converting enzymes in the ooplasm of C. versicolor ovary as reported in the present study
suggests the possibility of steroidogenesis although further evidence is needed to confirm this view.

Corpora lutea : Progesterone-like activity in the plasma and the ovaries of snakes (Bradyon 1954) and higher levels of plasma progesterone in the pregnant snakes than in the nonpregnant females of N. sipedon (Callard and Leathem, 1965) lend support to the view that corpora lutea of reptiles synthesize progesterone. According to Callard and his co-workers (1972) who investigated the cycle of progesterone level in blood of Sceloporus cyanogenys, corpora lutea are the major source of circulating progesterone. The results of the in vitro studies on N. sipedon and of the plasma progesterone in both pregnant and nonpregnant individuals of this species, are rather divergent. No virtual difference in the in vitro conversion of pregnanolone to progesterone by the ovary of both pregnant and nonpregnant N. sipedon was noticed (Callard, 1966). However, the plasma progesterone level in pregnant individuals of this species was higher than in the nonpregnant forms (Callard et al., 1972). Thus the consensus, though equivocal, tends to favour the view that the reptilian corpora lutea form the sites of biosynthesis of steroids, mainly progesterone. A similar conclusion has been arrived at from the histochemical
localization of lipids, cholesterol and its esters in corpora lutea of snakes and lizards (Guraya, 1976). The observations of in vitro conversion of pregnenolone to progesterone by the homogenate of the isolated corpora lutea of the snapping turtle, Chelydra serpentina serpentina (Klicka and Mahmoud, 1974) lend the conclusive evidence that the luteal bodies of reptiles are capable of steroidogenesis.

$\Delta^5-3\beta$-HSDH activity has been noticed in corpora lutea of some reptiles, N. sipedon (Callard, 1966); L. sicula (Callard et al., 1966), N. vigilis, N. caranus (Yaron, 1972) and A. carolinensis (Jones et al., 1972). The intense activity of steroid converting enzymes in the granulosa lutein cells of corpora lutea in C. versicolor, N. flaviviridis and C. calcaratus strongly suggests that the luteal bodies are involved in biosynthesis of steroid hormones. The presence of $17\beta$-HSDH, hitherto not reported in the corpora lutea of reptiles, further suggests that corpora lutea can synthesize sex steroids. Thus the presence of the steroid converting enzymes, namely $\Delta^5-3\beta$-HSDH, $17\beta$-HSDH, $11\beta$-HSDH and G-6-PDH, NADH diaphorase, ICDH, LDH enzymes and sudanophilic lipids in the luteinized granulosa cells of the corpora lutea in the ovaries of
C. versicolor, H. fleviviridis and C. calcaratus strongly suggest that corpora lutea of reptiles are involved in steroidogenic activity and that they possess the potency to synthesize progesterone, sex steroids and corticosteroids.

**Atretic follicles**: Follicular atresia is a feature found in all vertebrates and the atretic follicles are considered as the regressing elements of the ovary having a doubtful endocrinal role (Miller, 1959; Ingram, 1962; Lofts and Bem, 1972; Guraya, 1973, 1976; Saidapur, in press). Based on the incidence of follicular atresia and the appearance of secretory-like cells in theca interna, atretic follicles are adjudged as the possible source of estrogens in reptiles (Miller, 1959). On the contrary, absence of cholesterol or its esters in the atretic follicles has led to the conclusion that they may not be involved in steroid biosynthesis (Guraya, 1976). Thus it is not clear whether the atretic follicles of reptiles synthesize steroids or not. Out observations on the occurrence of steroid converting enzymes in the atretic follicles of C. versicolor, H. fleviviridis and C. calcaratus strongly suggest that they possess the steroidogenic potential. Polymorphic granulosa cells of atretic follicles develop transient steroidogenic activity that lasts till they are phagocytosed.
Further, patches of theca interna cells that contain steroid converting enzymes retain the steroidogenic activity even after atresia, till they are incorporated in the ovarian stroma as interstitial gland cells. \( \Delta^5-3\beta\)-HSDH has been demonstrated in the atretic follicles of other vertebrates, namely, fishes (Nadkarni, unpublished data), amphibians (Saadapur and Nadkarni, 1974), birds (Lofts and Bearn, 1972; references in Lofts and Hurton, 1973; Bhujle, 1977) and mammals (Rubin et al., 1963; Seth and Prasad, 1967). The presence of 17\( \beta\)-HSDH and 11\( \beta\)-HSDH activity in the atretic follicles in reptiles is to the best of our knowledge, the first report of its kind on the atretic follicles of the vertebrate series and the presence of these two enzymes suggests that the atretic follicles of reptiles are capable of biosynthesis of sex steroid and corticosteroids. Hence it would be of interest to carry out further studies using electron microscope and in vitro and in vivo conversion of steroids by the isolated atretic follicles in reptiles.

Ovarian stroma: The presence of \( \Delta^5-3\beta\)-HSDH, 17\( \beta\)-HSDH and 11\( \beta\)-HSDH enzymes in the interstitial gland cells of the ovarian stroma in C. versicolor, H. flaviviridis and C. calcaratus, not reported till now, reveals that these cells form the site of biosynthesis of \( C_{21}, C_{19}, C_{18} \) steroid
hormones and corticosteroids. Further the presence of intense $\Delta^5-3\beta$-HSDH activity in the cells suggests that the ovarian interstitial cells contribute significant amount of steroids to the ovarian steroid pool. Histochemical observation of the steroid converting enzymes throughout the process of atresia in the ovaries of the three lizards suggests that the interstitial gland cells may arise from the cells of theca interna of atretic follicles. This observation supports the earlier studies based on the histological observations and histochemical demonstration of sudanophilic lipids, cholesterol and its esters in the interstitial cells (Guraya, 1965, 1973, 1976; Vamsi and Guraya, 1973 a,b). A relatively moderate reaction for $17\beta$-HSDH in the interstitial cells might signify their role as internal secretor of estrogens and androgens. Similarly the ability of these cells to synthesize corticosteroids may be inferred from the presence of $11\beta$-HSDH activity in the lizards investigated in the present work. It is interesting to note here that the ovarian stroma of some mammals is known to synthesize androgens and corticosteroids (Richardson, 1963).

**Cyclic activity**: It was not possible to make a subjective estimation of the enzyme activity in the ovary as a whole from the monthly histochromical studies, since some of the
ovarian elements that showed steroidogenic potential were either transient in nature or else underwent changes that amounted to increase or decrease in the number of steroidogenic cells. The number of stromal interstitial cells, the number of atretic follicles and the postovulatory follicles were not disposed uniformly so that an over-all activity of the ovary could be judged from the observations of a few frozen sections. However, some broad-based generalizations could be made from the monthly histological and histochemical studies.

An increasing number of interstitial cells concomitant with the increase in the bulk of the ovarian stroma in *C. versicolor* during the onset of regeneration in January and the increased development of the ovary till the breeding phase is over in August indicate, though indirectly, that the ovarian stroma is the major source of circulating steroid hormones, estrogen and progesterone needed for stimulating the genital ducts into activity. A number of studies presently show that estrogens and progesterone bind with proteosaceous receptors present in the oviducts and that these steroid hormones induce the oviducts into activity during breeding (Ozon, 1972; Chester Jones et al., 1972).

During the breeding and postbreeding periods from May to December, the postovulatory follicles may contribute
to the ovarian steroidogenesis, as these luteal bodies remain secretory in function till December. The active condition of the corpora lutea persist till the oviposition of the last clutch of eggs in *C. versicolor*. Thus corpora lutea of lizards might contribute a significant amount to the steroid titre of the blood. A number of studies has shown that egg retention and longevity of corpora lutea in reptiles are interrelated (Miller, 1959) and progesterone is essential for maintenance of eggs in oviducts (Miller, 1959; Yaron, 1972; Guraya, 1976). The precise role of the reptilian corpus luteum is by no means clear and conflicting evidences have been presented on the necessity of corpora lutea in the maintenance of gestation in viviparous snakes (Amoroso and Finn, 1962). It is inferred from an indirect evidence that corpora lutea inhibit ovulation rather than bring about progestational changes in the uterus of viviparous reptiles (Cunningham and Smart, 1934). Corpora lutea together with the hypophysis seem to be necessary in early pregnancy in viviparous snakes (Rahn, 1939; Clausen, 1940; Fraenkel et al., 1940). Hypophysectomy prior to ovulation in *Zootoca vivipara* (Panigel, 1956) and *Sceloporus cyanogenys* (Callard and Ziegler, 1970; Callard et al., 1972b) results in atrophy of ovary and oviduct. Hypophysectomy during gestation will not lead to abortion but will interfere with parturition as manifested
by the retention of the embryo in utero past full term. The interference is more with hypophysectomy than with ovariectomy. Thus the mechanism by which this is brought about remains unclear.

Much needs to be elucidated about the hormonal regulation of the ovarian functions in reptiles (Miller, 1959; Amoroso and Finn, 1962; Callard et al., 1972; Yaron, 1972). Several experiments have been carried to study the effect of exogenous estrogens, PMS (pregnant mare serum) and growth hormones on the reptilian ovary. These studies tend to indicate that estrogen is essential for vitellogenesis and estrogen synthesis in the reptilian ovary is inhibited by progesterone (Callard et al., 1972). Thus progesterone synthesized mainly in the corpora lutea might play an antagonadal role in inhibiting biosynthesis of estrogens needed for vitellogenesis and ovulation. The experiments in which intra-hypothalamic implants of estrogens were made, provide a good evidence of estrogenic feedback inhibition of pituitary gonadotropin release leading to failure of steroid production and ovulation (Lisk, 1967). The effect of progesterone implant in the hypothalamus manifested in the prevention of vitellogenesis, induced follicular atresia and regression of oviducts (Callard et al., 1972b).
In the light of earlier studies by other workers, designed to elucidate the hormonal regulation of the ovarian functions in reptiles, our observations on the cyclic changes in the activity of steroid converting enzymes in the ovary in C. versicolor, becomes meaningful. A high activity of enzymes in the ovarian stroma during regeneration and breeding phases, January to August, might signify an increase in estrogen synthesis resulting in rapid growth and vitellogenesis of the follicles. During the period from the beginning of breeding (May) till the oviposition of last clutch of eggs (December), the high level of progesterone as judged from the number of active corpora lutea, might reflect (1) drop in the estrogen level, (2) prevention of ovulation and (3) initiation of follicular atresia during the later half of the breeding phase and the entire period of regression (July-December).

**SUMMARY**

1 The activity of $\Delta^5-3\beta$-HSDH, 17$\beta$-HSDH, 11$\beta$-HSDH, G-6-PDH, NADH diaphorase, LDH, ICDH enzymes and lipids in patches of cells in theca interna of normal and atretic follicles, granulosa of large preovulatory and atretic follicles in early stage,
granulosa luteal cells of postovulatory follicles, interstitial cells of the ovarian stroma and in the oocyte of vitellogenic normal follicles, suggests that ovarian elements in *C. versicolor*, *H. leviriridis* and *C. calcaratus* are involved in the biosynthesis of steroid hormones.

2 The presence of 17β-HSD in these ovarian elements indicates their ability to synthesize estrogens and androgens.

3 The occurrence of 11β-HSD suggests that the ovary of the three lizards is capable of biosynthesis of corticosteroids, 11β-estrogens and 11β-androgens.

4 The reptilian oocyte has been observed to show weak but positive steroidogenic potency as judged by the presence of steroid converting enzymes.

5 Granulosa cells of the atretic follicles possess transient steroidogenic activity that lasts till these cells are phagocytosed.

6 The theca interna cells of atretic follicles that contain steroid converting enzymes transform into interstitial gland cells of the ovarian stroma.
Corpora lutea in the ovaries of the lizards appear during the breeding phase (May-August) and continue up to the end of the regression period (September-December).

The granulosa lutein cells of the postovulatory follicles (luteal bodies) are capable of steroidogenesis as revealed by the presence of steroid converting enzymes in them.

The increased number of stromal interstitial cells during the periods of regeneration (January-April) and breeding (May-August) may contribute significant amount of steroids to the ovarian steroid pool.
Table 3.1: \( \Delta^5-3 \beta\)-HSDH, 17\( \beta\)-HSDH, 11\( \beta\)-HSDH, ICDH, LDH, G-6-PDH and NADH diaphorase activity in the ovaries of *C. versicolor*, *H. flaviviridis* and *C. calcarius*.

<table>
<thead>
<tr>
<th>Enzymes and substrates</th>
<th>Normal Follicles</th>
<th>Corpora Lutea</th>
<th>Atretic Follicle</th>
<th>Interstitial gland cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theca interna</td>
<td>Granulosa</td>
<td>Gooocyte</td>
<td>Theca interna</td>
</tr>
<tr>
<td>( \Delta^5-3 \beta)-HSDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>17( \alpha)-CH pregnenolone</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>DHEA</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Stiocholanolone</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>17( \beta)-HSDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>17( \beta)-estradiol</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>11( \beta)-HSDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11( \beta)-hydroxy androstanedione</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ICDH, LDH, G-6-PDH &amp; NADH DIAPHORASE</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Control</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Activity is graded from '+' to maximum, '+++++' intensity. Coenzyme NAD was used for all enzymes except for G-6-PDH, and ICDH. All chemicals are of Sigma grade obtained from Sigma Chemical Company, St. Louis, U.S.A.
EXPLANATION TO FIGURES

Fig. 3.1  LDH activity in the ovary of *S. versicolor*. Part of the large ovulatory follicle showing the activity along the periphery of the ooplasm (O) and patches of cells in theca interna (TI). The activity in the ooplasm of small follicles is spread out. X100.

Fig. 3.2  NADH diaphorase in the ovary of *C. calcaratus*. Part of the large preovulatory follicle showing the activity in ooplasm (O), granulosa (G) and theca interna (TI). X200.

Fig. 3.3  ICDH in the ovary of *S. versicolor*. The activity is seen towards the periphery of ooplasm (O), in the granulosa (G) and theca interna (TI). X200.

Fig. 3.4  $\Delta^5$-3/?-HSDH in the ovary of *S. versicolor*. Normal follicle (NF) showing granulosa devoid of activity. The other follicle is an atretic follicle (AF) in which granulosa showing intense activity have migrated into the ooplasm. X200.
EXPLANATION TO FIGURES

Fig. 3.5 Small follicles in the ovary of *H. flaviviridis* showing LDH activity seen in ooplasm varying with the size of the follicles. Part of the normal follicle (NF) prior to ovulation is also seen. X100.

Fig. 3.6 Atretic follicle in the ovary of *C. versicolor* undergoing glandular atresia. Intense G-6-PDH activity is seen in granulosa (G) and theca interna (TI). Note the collapsing vitelline membrane (V) and the disorganised ooplasm (O). X100.

Fig. 3.7 Follicle of *C. versicolor* ovary undergoing yolk atresia. $\Delta^5$-3$\beta$-HSDH is seen in granulosa (G) that have invaded the ooplasm (O). DHSA is the substrate. X30.

Fig. 3.8 $\Delta^5$-3$\beta$-HSDH in the ovary of *H. flaviviridis*. The activity is seen in granulosa cells invading the ooplasm of yolky atretic follicle. X100.
EXPLANATION TO FIGURES

Fig. 3.9 Ovary of *H. flaviviridis* showing yolky atresia. 11β-HSDH activity is seen in the granulosa cells (G) invading the oocyte. X100.

Fig. 3.10 Large follicle in the ovary of *C. calcaratus* undergoing glandular atresia. Intense 5α-3β-HSDH activity is seen in granulosa cells (G) forming wavy folds. DHEA is the substrate. X100.

Fig. 3.11 Atretic follicle of *C. calcaratus* showing 17β-HSDH activity in the granulosa (G) forming wavy folds. Testosterone is the substrate. X100.

Fig. 3.12 Ovary of *H. flaviviridis* showing glandular atresia. 5α-3β-HSDH activity is seen in the granulosa cells (G) that have phagocyted the ooplasmic contents. DHEA is the substrate. X100.
EXPLANATION TO FIGURES

Fig. 3.13  G-6-PDH activity in the atretic follicle of *C. calcaratus* showing intense activity in granulosa cells. The activity is also seen in small patches of cells in theca interna (TI), X100.

Fig. 3.14  Newly discharged postovulatory follicle of *C. versicolor* showing intense ICDH activity in luteinised granulosa cells (G). X40.

Fig. 3.15  G-6-PDH activity in the luteal cells (G) of the postovulatory follicle in *C. versicolor*. Part of the normal follicle showing the activity in theca interna (TI) is also seen. X40.

Fig. 3.16  Reaction for $\Delta^3$-3-ketosteroid with diäsa as the substrate is obtained in granulosa luteal cells of *C. versicolor*, X40.
EXPLANATION TO FIGURES

Fig. 3.17  NADH diaphorase activity in corpora lutea (CL) of *G. calcaratus*, X40.

Fig. 3.18  \( \Delta^5-3 \beta \)-HSDH activity by using DHEA in the corpora lutea of *G. calcaratus* ovary, X40.

Fig. 3.19  Lipids in granulosa lutein cells of the corpus luteum of *H. flaviviridis* (Oil Red O method), X40.

Fig. 3.20  \( \Delta^5-3 \beta \)-HSDH activity in the interstitial stromal cells (SC) in the ovarian stroma of *G. versicolor*. DHEA is the substrate used, X40.
Fig. 3.21 Ovary of C. versicolor showing \( \Delta^5-3\beta \)-HSDH activity in the interstitial gland cells (IG). Portion of the normal follicle (NF) is seen. X40.

Fig. 3.22 Intense \( \beta \)-HSDH activity in the ovarian stroma of H. flaviviridis. Testosterone is the substrate and NAD is the coenzyme, used. X40.

Fig. 3.23 Ovarian stroma of H. flaviviridis showing the \( \Delta^5-3\beta \)-HSDH activity in the interstitial cells. Degenerating luteal bodies (CA) do not show reaction for this enzyme. X40.

Fig. 3.24 Ovarian stroma of C. versicolor showing \( \Delta^5-3\beta \)-HSDH activity. The stroma shows small follicles undergoing atresia. X40.