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HISTOCHEMICAL STUDIES OF ENZYMES INVOVED IN HORMONAL REGULATION IN GARDEN LIZARD (Calotes versicolor)

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Abstract: **Studies in situ changes in various enzyme activities viz. Δ^5-3β-HSDH, Peroxidase, Acid and Alkaline phosphatases, Cytochrome oxidase &Lipids in the adrenal gland and ovary at different stages of reproductive cycle in Garden Lizard (Calotes versicolor). Peroxidase appears to be involved in the biosynthetic machinery controlling corticosteroidogenesis. Peroxidase and Cytochrome oxidase would also seem to transform adrenocortical cells and hypertrophied theca interna into highly oxidative compartments of the adrenal and ovary which attributes to the oxidation of pregnenolone to progesterone and corticosteroids towards maturation and ovulation of the oocyte from the ovary.**

Keywords: **Biosynthetic Machinery, Enzymes, Adrenal-Ovary Interrelation & Pregnenolone to Progesterone, Corticosteroidogenesis.**

1. INTRODUCTIION

The various in vivo and in vitro studies have demonstrated that the ovary and adrenal possess the side-chain cleaving system to convert C_{27} cholesterol to pregnenolone which are mainly a C_{22}-C_{20} lyase and hydroxylases (Simmer, 1968). Other Steroidogenic enzymes viz. sulfatase, steroid -isomerase, 3B-OH-steroid dehydrogenase and 20-α-hydroxysteroid dehydrogenase have also been reported (Beyer et al., 1956; Burstein et al., 1963 and Weist et al., 1963) which are involved in the biosynthesis of progesterone and androgens.

The sex hormones produced by the adrenal cortex of both males and females are progesterone, testosterone and estrogens. The adrenal gland is the source of sex hormones until the testis and ovaries mature at puberty. The secretion of these hormones is controlled by ACTH and not by gonadotrophins which stimulate the testes and the ovaries.

Since ,adrenals are known to secrete large quantities of progesterone, which is an oxidation product Of pregnenolone, it appears probable that conversion of pregnenolone to progesterone may be brought about peroxidatively by the operation of peroxidase as suggested in the ovary (Agrawal and Laloraya, 1977). The role of peroxidase in the endocrine regulation of hormone action in the adrenals which is closely interlinked in reproductive functioning of different groups of animals remains largely unknown. There appear important gaps in the understanding of the hormone regulation and the enzymic mechanis which lead to the rapid formation and secretion of hormones namely progesterone and corticosteroids in the adrenal gland is largely unknown.

A relationship between adrenal steroidogenesis and reproduction has been demonstrated for several species (Christian, 1963; Liptrap, 1970; Ramaley, 1973). Progesterone is known to be a precursor of several steroid hormones including androgens, estrogens corticoids..Samuels and Uchikawa (1967) in In Vitro studies have shown that it occupies a key position in the biosynthesis of adrenal corticoids. The presence of Δ^5-3β- hydroxysteroid dehydrogenase, 11β-hydroxysteroid dehydrogenase, 17 β – hydroxysteroid dehydrogenase and glucose-6-phosphate dehydrogenase was demonstrated histochemically in the adrenal glands of some birds. All these enzymes occurred in the interregnal cells and no activity was observed in the chromaffin cells. The interregnal cells are probably capable of synthesizing both corticosteroids and sex steroids (Bhujle et al., 1976).

The enzyme activities like Δ^5-3β-hydroxysteroid dehydrogenase, 21 Hydroxylase(21-OHase) and 11β-hydroxylase (11β-OHase) in the adrenals of mammals have been correlated with the synthesis of progesterone and corticosteroids in the cells of zona fasciculata and zona reticularis (Rubin et al.,1957; Chester Jones, 1957 and Rubin et al., 1963). ACTH is shown to promote Cholesterol ester hydrolysis by activating cholesterol esterase (Tyslowitz, 1943; Long 1947) thereby stimulating the synthesis of pregnenolone ,the intermediate precursor of progesterone. How this pregnenolone is rapidly converted to progesterone in adrenals remains to be known. In biological systems a group of enzymes known as the peroxidases are known to bring about rapid oxidations of organic molecules using H_2O_2. The importance of this reaction in regulating steroidogenesis has received active attention (Agrawal & Laloraya ,1977,1980; Agrawal & Harper,1982;Lyttle&Jellinck,1976andDeme et al., 1978). LH is shown to promote cholesterol ester hydrolysis by activating cholesterol esterase(Behrman and Amstrong , 1979) , this stimulates net progesterone secretion by increasing conversion of cholesterol to pregnenolone. Also it stimulates the synthesis of the enzyme, Δ^5-3β-hydroxysteroiddehydrogenase which plays a key role in the early biosynthetic pathways of all the biologically active steroid hormones.

The objective of the present investigation therefore was:

1. To study in situ changes in various enzyme activities viz. Δ^5-3β Hydroxysteroid dehydrogenase, Peroxidase, acid and alkaline phosphatases and cytochrome oxidase in Garden Lizard (Calotes versicolor).

2. To work-out the biochemical mechanism controlling steroid biogenesis.

3. To understand the Physiological importance of adrenal progesterone which is yet to be known.

2. MATERIAL & METHODS

The experimental animal , Garden Lizard (Calotes versicolor)were supplied by an animal catcher at different reproductive phases They were sacrificed at one fixed time keeping in mind the circadian rhythm in the secretions of the hypothalamo-hypophyseal-adrenal axis as reported by a large number of workers (Ganong, 1963 ; Critchlow, 1963; Critchlow et al., 1963) and later subjected for histochemical studies.

Histochemical Procedure: Gelatin fixed frozen sections (4µ) of the interregnal tissue were cut in the American Optical Cryocut and were then used for the localization of various enzymes.

1. Peroxidase: This enzyme was localized by the modified method of Van Duija (1951) using benzidine as a donor. Another method Graham &Karnovsky (1966) using diaminobenzidine as a donor was alsoapplied.The activity was also tested with the other donor namely Guaiacol.

2. Cytochrome Oxidase: The method followed for the localization of Cytochrome oxidase was that of Burstone, (1959).

3. $\Delta^5$3β- Hydroxysteroid dehydrogenase: This enzyme was localized by the method of Wattenberg(1958).

4. Acid Phosphatase: This enzyme was localized after the method of Gomori,(1950).

5. Alkaline Phosphatase: The Calcium-Cobalt method for Alkaline phosphatase , Gomori (1952) wasfollowed .

6. Lipids: Lipids were stained in frozen sections (4µ) by Herxheimer's fat stain method (1903).

3. RESULTS

3.1 Adrenal Gland

Adrenal gland of reptiles undergoes seasonal changes in histology and histochemistry of the cortical tissue. The cortical tissue consists of polyhedral low columnar cells that are not arranged in definite cords. These cells have round or oval basophilic nuclei, the appearance of which vary seasonally and become round and turgid during the secretory stage. The subcapsular and inner cortical zones of the adrenal undergo marked seasonal fluctuations throughout the breeding season.

3.2 Δ^5-3β- Hydroxysteroid Dehydrogenase

A positive Δ^5-3β- Hydroxysteroid dehydrogenase has been seen in the inner cortical cells of the follicular stage (Plate 1A) which increases during ovulatory phase (Plate 1B). A decline in activity could be observed during the post-ovulatory phase (Plate 1C). the increased activity of this enzyme in cortical adrenal elements is apparently related to the corticosteroids production.

3.3 Cytochrome Oxidase

A moderate activity of Cytochrome oxidase is seen during the follicular phase (Plate2A) which increases during the ovulaory phase (Plate2B). A sharp decline in activity is observed during the post-ovulatory phase (Plate 2C).

3.4 Peroxidase

The histochemical properties of the reptilian adrenocortical tissue is more or less similar to those of mammals. Peroxidase is present in the inner cortical tissue during the ovulatory phase (Plate 3B)while diffused activity is seen during the post-ovulatory period (Plate 3C).. the enzyme is distributed irregularly throughout the cytoplasm. Low activity of peroxidase is seen during the follicular phase Plate 3A).

3.5 Acid and Alkaline Phosphatases

The cortical cells show high activity of acid phosphatase during the ovulatory phase (Plate 4B), which gets diffused during the post-ovulatory phase (Plate 4C). During the follicular stage, the activity appears to be weak compared to other stages (Plate 4A). Alkaline phosphatase showed a high activity during both ovulatory and post-ovulatory stages (Plate 5A&B).

3.6 Lipids

In contrast to other enzymes which show a high activity during the ovulatory phase, the deposition of lipid is decreased at the ovulatory and post-ovulatory phase (Plate 6B & C) with high content at follicular Phase (Plate 6A).

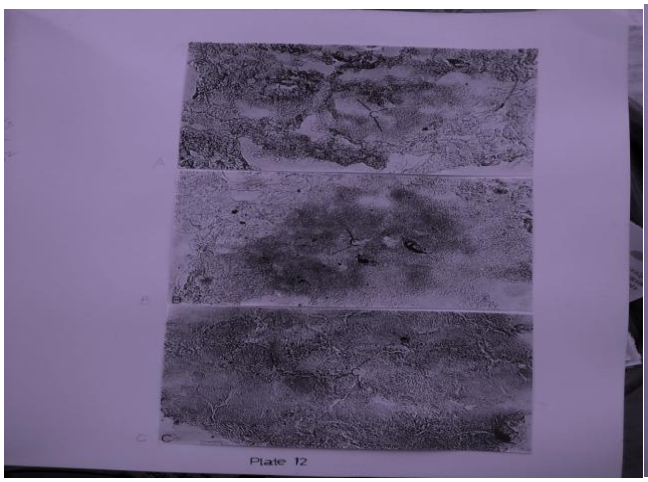

Plate 1A,B & C

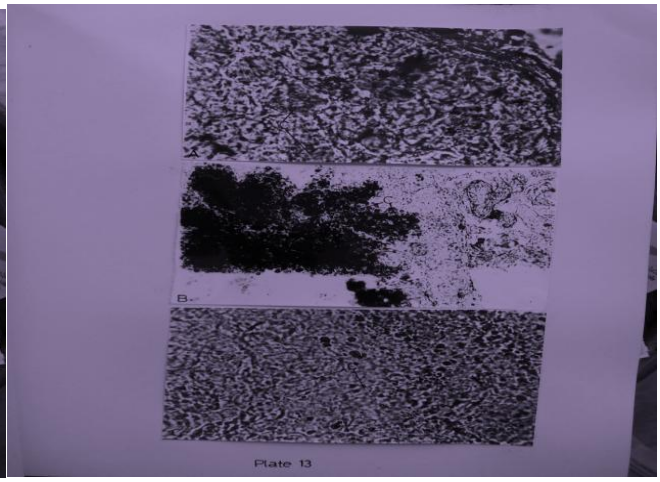

Plate 2A,B & C

Localization of enzymes in the Adrenal Gland of Garden lizard(Calotes) at different phases of Sexual cycle – Plate 1 -> Δ^5-3β- Hydroxysteroid dehydrogenase & Plate 2 -> Cytochrome oxidase

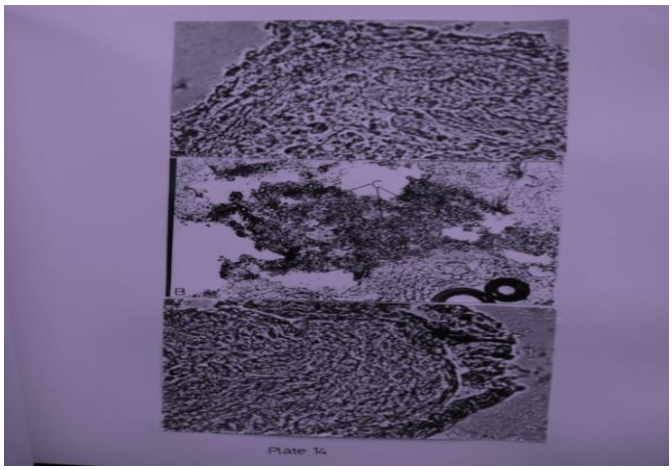

Plate 3 A,B & C

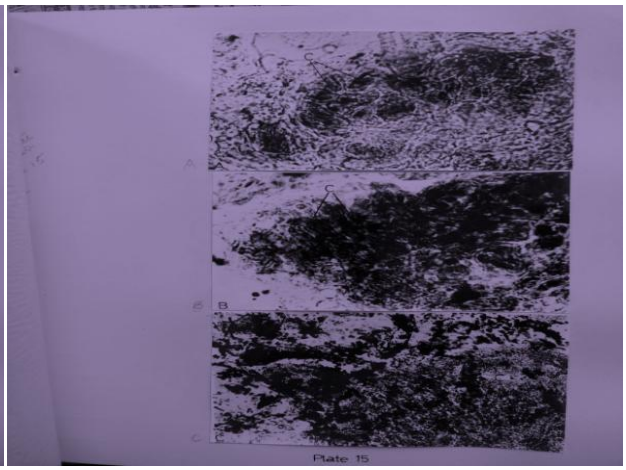

Plate 4 A,B & C

Localization of enzymes in the Adrenal Gland of Garden lizard (Calotes versicolor) at different stages of reproductive cycle -Plate 3 ->Peroxidase & Acid phosphatase (Follicular ,Ovulatory & Post- Ovulatory phases). Plate 4-> Acid phosphatase(follicular, Ovulatory & Post-Ovulatory phases).

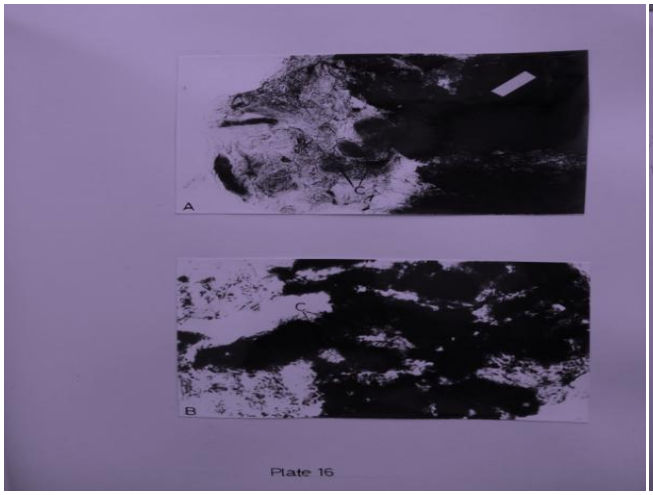

Plate 5 A & B

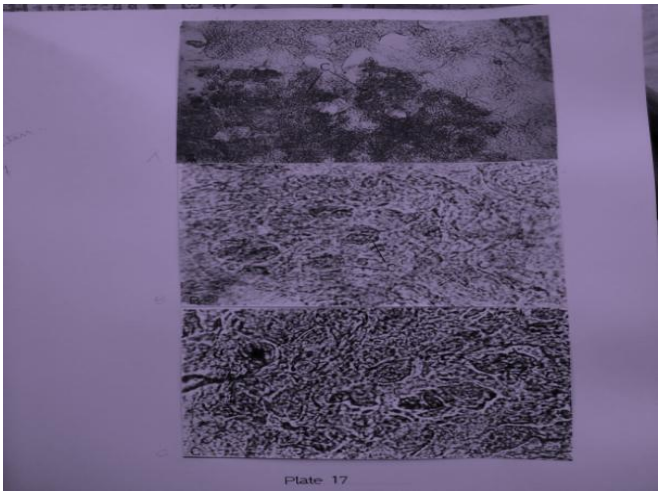

Plate 6A,B & C

Localization of enzymesin the Adrenal of Garden Lizard (Calotes versicolor)at different stages of sexual cycle. Plate 5 -> Alkaline phosphatase(ovulatory & Post-Ovulatory phase) & Plate 6 -> Lipids (Follicular,Ovulatory & Post-Ovulatory phases.

4. OVARY

4.1 Follicular Growth

The developing oocyte in the reptilian ovary ia associated with theollicularpithelium(pleomorphicGranulosa cells) and thecal layer. The ovarian stroma is well developed.. No activity of peroxidase could be observed in the growing follicles(Plate 7A). A high localization of Δ^5-3β- Hydroxysteroiddehydrogenase is seen in the IGT (Plate 7B). Cytochrome oxidase activity also is high at this stagein the IGT (Plate 7C). Acid phosphatase activity is seen to be high in the growing oocytes and in IGT (Plate 7D).

Growing follicles show an intense activity of alkaline phosphatase at this stage (Plate 8A). Control section without the substrate show no activity (Plate 8B).

Sudanophilic lipid deposition is shown to be high in the IGT (Plate 8C). Control section does not show any activity (Plate 8D).

As the follicles grow in size the surrounding stromal tissue becomes organised in the form of fibrous theca interna. The well developed cytoplasmof the thecal cells show high Peroxidase, Δ^5-3β- Hydroxysteroid dehydrogenase and Cytochrome oxidase activity (Plate 9A,B & C), while diffuse localization of Δ^5-3β- Hydroxysteroid dehydrogenase ans Cytochrome oxidase is seen in the granulosa cells (Plate 9B & C). The IGT are well developed in reptiles during ovulatory phase which exhibits

ntense activity of Δ^5-3β- Hydroxysteroiddehydrogenase and Cytochrome oxidase (Plate 9A,B & C) but with no peroxidase activity. The black patches are seen due to the accumulation of brown-yellowlipid substances.

The well vascularized thecal cells at this phase show high activity of AcidPhosphatase (Plate 10A) and Alkaline phosphatase (Plate 10B). A diffuse localization of Acid phosphatase is seen in granulosa cells (Plate 10A).

Lipid droplets are high in the IGT (Plate 10C & D) with diffuse localization in the membrana granulosa (Plate 10C).

4.2 Post-Ovulatory Follicles:

Calotes are are oviparous and develop true post-Ovulatory corpora lutea which are formed from the hypertrophy of granulosa and thecal cells. These cells are well vascularized. The most striking and significant change during the transformation of granulosa cells, is the appearance of very high Peroxidase and Cytochrome oxidase activity throughout the cytoplasm of the later. Post-ovulatory follicles show heavily stained CL with no activity in IGT (Plate 11A & B).

Diffuse localizatiion of Δ^5-3β- Hydroxysteroid dehydrogenase is seen in the CLand IGT (Plate 11C). the atretic follicles also show weak localization of Δ5-3βHydroxysteroid dehydrogenase(Plate 11C).

Acid phosphatase and Alkaline phosphatase activity is high in the transformed luteal cells (Plate 12A & B) with weak localization of Acid phosphatase in theMembrana granulosa of atretic follicle. The activiy of lLipid decreases in the the Luteal cells and IGT (Plate 12C).

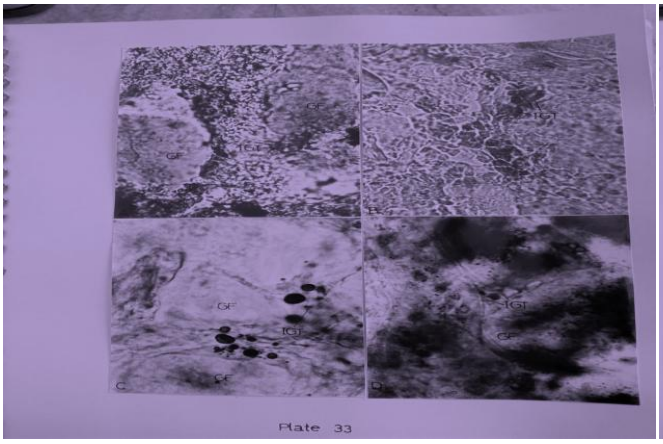

Plate 7A,B,C & D

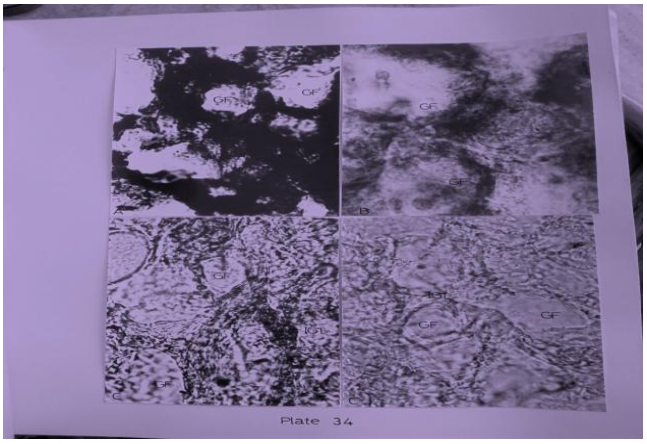

Plate 8A,B,C & D

Localization of enzymes in the Ovary of Garden Lizard (Calotes versicolor) at Follicular *Phase*. Plate 7-> Peroxidase , Δ^5-3β- Hydroxysteroid dehydrogenase, Cytochrome oxidase & Acid phosphatase & Plate 8 -> Alkaline phosphatase &Lipids (B & D – Control sections against A & C)

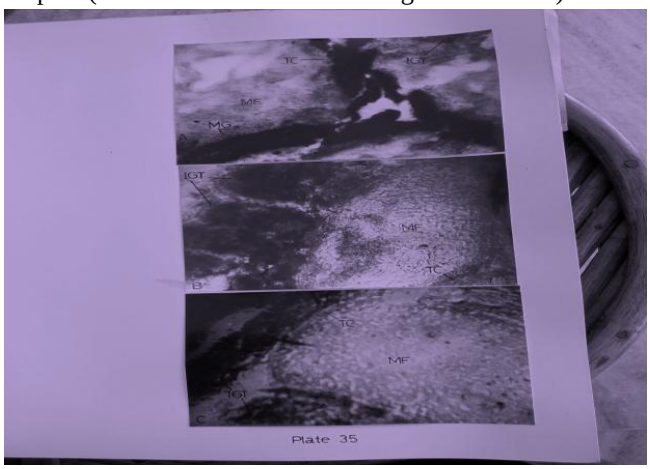

Plate 9A,B & C

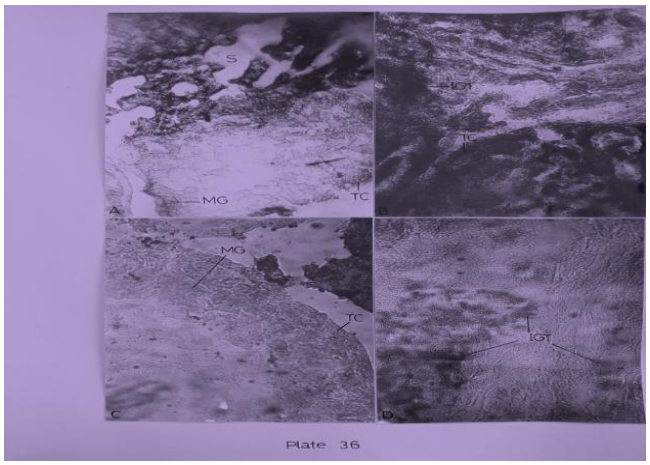

Plate 10A,B,C & D

Localization of enzymes in the ovary of Garden Lizard (Calotes versicolor) at Ovulatory Phase Plate 9 -> Peroxidase,Δ^5-3β- Hydroxysteroid dehydrogenase & Cytochrome oxidase & Plate 10 -> Acid phosphatase, Alkaline phosphatase and Lipids.

Plate 11A,B & C

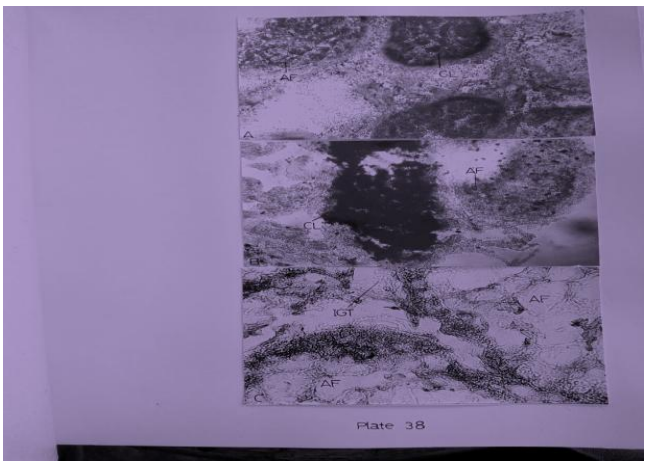

Plate 12A,B & C

Localization of enzymes in the ovary of Garden Lizard (Calotes versicolor) AT Post – Ovulatory phase of the sexual cycle. Plate 11 -> Peroxidase ,Cytochrome oxidase & Δ^5-3β- Hydroxysteroid dehydrogenase & Plate 12A,B & C -> Acid phosphatase,Alkaline phosphatase & Lipids.

5. DISCUSSION

peroxidase is present in the inner layer of adrenocorticalCells but not in chromaffin cells of ovulatory animals of different groups of vertebrates which are associated with the functioning of ACTH hormone and progesterone,corticosteroid secretion.The Δ^{5}-3β – Hydroxysteroid dehydrogenase and Cytochrome oxidase are present in the adrenocortical cells during the entire period of sexual cycle.Thus the characteristic function of adrenocortical cells regulating large secretion of progesterone during increased sexual activity appears to be related to the presence of peroxidase in these ompartments.The adrenal cortex of many non-mammalian species are recognized as the chief site of conversion of ^{14}C acetate to progesterone (Vinson and Whitehouse, 1973a). Also the biochemical studies have shown that adrenal cortex is the chief site for the synthesis of steroid hormones namely Progesterone, cortisol and corticosterone (Hayano et al., 1956; Resko, 1969; Holzbauer, 1969) .

Histochemical studies in fish have shown the presence of Δ^{5}- 3β-Hydroxysteroid dehydrogenase, 11β- Hydroxysteroid dehydrogenase,17β- Hydroxysteroid dehydrogenase and G-6- PDH in the interrenal cells (Hooli et al., 1974; Hooli et al., 1976; Bhujle et al.,1980). Δ^5 - 3β – Hydroxysteroid dehydrogenase being present during the entire sexual cycle viz., follicular,prespawning and spawning period,the specific function of the adrenocortical cells appears to be characterized by the presence of peroxidase ,which is observed in the cortical cells alone during the ovulatory phase and pregnancy, the well known site for the secretion of Progesterone and corticosteroids . The granulosa cells of developing follicles, corpora atretica and oocytes in the ovaries of some species of fishes, amphibians and reptiles have been shown to develop enzyme activities indicative of steroidogenesis (Guraya,1971, 1972a; Rubin et al.,1963; Deane et al.,1962; Lobel & Levy, 1968). Presence of high peroxidase activityin the hypertrophied theca interna of ovulatory follicles and also the CL after ovulation in frog suggest that basic factor involved in leutinization of granulosa cells and also progesterone synthesis are the same for mammalian and non-mammalian vertebrates and that peroxidase appears to be one of the common factors involved in both. Progesterone and corticosteroids of adrenal origin has been attributed a function in ovulation and spawning in non-mammalian vertebrates namely fish and amphibian (Sundararaj and Goswami, 1966b; Goswami and Sundararaj, 1971) . The presence of high peroxidase activity in the adrenocortical cells of the non-mammalian vertebrates during the ovulatory phase may be correlated with the synthesis of progesterone which act synergistically with corticosteroids to cause ovulation in these species as suggested in the fowl (Soliman et al., 1974).

Furthermore, since peroxidase mediated reactions are many fold faster than dehydrogenase reactions, the association of high peroxidase activity in these regions, and lack of activity in adrenocortical cells at follicular phase, in growing follicle of the ovary and IGT of the ovary would suggest that the high rate of progesterone formation may be associated with the functioning of this enzyme at specific sites. Peroxidase thus appears to be involved in the biosynthetic machinery controlling corticosteroidogenesis.

The histochemical changes in acid and alkaline phosphatases and lipids in the adrenocortical cells and ovary at various reproductive phases have been shown by a number of workers (Galli Mainini, 1951; Botte, 1964) .High acid phosphatase activity is shown to be present at ovulatory Furthermore phase in the adrenocortical cells and ovary of fish, amphibians and reptiles, while alkaline phosphatase attains zenith during the secretory phase (spawning phase) . Sudanophilic granules have been shown to increase markedly in the adrenocortical cells,TI and IGT of the ovary At the follicular phase and disappears during the spawning period. Under the hormonal stimuli (Guraya ,1974) the marked decrease in the lipids in ovary with increase in acid and alkaline phosphatase activity of spawning period confirm these reports.

The presence of active cytochrome oxidase in the adrenocortical cells and TI,CL,IGT of the ovary is suggestive of high metabolic activity in these tissues. The operation of active Cytochrome oxidase suggest that the necessary respiratory energy in the form of ATP molecules for the biosynthesis of lipids would be available at the site.The hypertrophied TI and CL of non-mammalian vertebrates are characterized by high vascularization and increased bloodFlow is also visible in adrenocortical cells of all vertebrates. The oxidative sites thus provided with adequate Oxygen supply with the activated blood flow thus converting thesesites into intense oxidative sites and thus the intense Cytochrome oxidase activity in these sites becomes meaningful. The Peroxidase and Cytochrome oxidase would also seem o transform adrenocortical

cells and hypertrophied TI into a highly oxidative compartment of the adrenal and ovary which attributes to the oxidation of pregnenolone to progesterone and corticosteroids towards maturation and ovulation of the Oocyte from the ovary.

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Abbreviations: AF- Atretic Follicle, CL- Corpus luteum, GF- Growing follicle, IGT- Interstitial gland tissue, LC – Luteal cells, MF – Mature follicle.

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