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HISTOCHEMICAL OBSERVATIONS OF ENZYMES DURING ESTROUS CYCLE IN THE ADRENAL GLAND OF RAT (Wistar rat)

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Abstract: **Peroxidase appears to be involved in the biosynthetic machinery controlling corticosteroidogenesis Peroxidase and Cytochrome oxidase would seem to transform adrenocortical cells into a highly oxidative compartments of the adrenal which attributes to the oxidation of Pregnenolone to Progesterone and Corticosteroids towards maturation. In view of this, a study of in situ changes in various enzymes viz.Δ53β-Hydroxysteroid dehydrogenase, Peroxidase, Cytochrome oxidase, Acid & Alkaline phosphatases & Lipids in the adrenal gland at different stages of reproductive cycle in Rat (Rattus rattus) had been studied.**

Keywords: **Enzymes, Estrous Cycle, Adrenal, Pregnenolone to progesterone & Biosynthetic machinery.**

I. INTRODUCTION

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). Steroidogenic enzymes such as 3B-OH-steroid dehydrogenase and 20 -hydroxysteroid dehydrogenase have 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 mechanism which leads 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 (Fig. 1).Samuels and uchikawa (1967) in Vitro studies have shown that it occupies a key position in the biosynthesis of adrenal corticoids. The physiological importance of adrenal progesterone in the rodent or any other species is not yet known. How this pregnenolone is rapidly converted to progesterone in adrenals remains to be known.

The objective of the present investigation therefore was:

To study in situ changes of various enzyme activities viz. Δ^5-3β-Hydroxysteroid dehydrogenase,Peroxidase, Acid andAlkaline phosphatases,Cytochrome oxidase & Lipids in adrenal ofRat (Rattus rattus) during estrous cycle so as to understand the physiological importance of adrenal progesterone In Situ changes of various enzymes involved in the peroxidative pathways of steroid biogenesis.

II. MATERIAL & METHODS

Colony-bred albino rats (Wistar Strain) of our departmental colony maintained on a regimen of 12 hrs. light/12 hrs. dark in a temperature controlled room (25^{0} - $^{+}1^{0}$ C) were used in this study.

They received food & water ad labium. Vaginal smears were taken daily and at least two complete cycles were observed in each rat prior to its use in an experiment. The mature female rats used for the study showed a regular 4-5 days days estrous cycle. The adrenal of sexually mature rat were used.

Histochemical Procedure: Gelatin fixed frozen sections (4µ) of the adrenal gland were cut in an 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 Benzidinas as donor. Another method Graham &Karnovsky (1966) using diaminobenzidine as a donor was also applied.Similar pattern was obtained with this donor. Therefore, benzidine as donor was used in the histochemical tests of peroxidase. 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. Δ^{5}3β- Hydroxysteroid dehydrogenase: This enzyme was localized by the method of Wattenberg.

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) was followed .

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

III. RESULTS

Histochemical localization of enzymes in the Adrenal Gland:

Peroxidase: It is detectable in the zona fasciculate and zona reticularis at estrous phase of the cycle.(Plate 1A), when guaiacol was used as a donor. A diffuse activity of this enzyme is seen in the bloodvessel walls of the chromafin cells. A high activity of peroxidase is seen in the zona fasciculate and zona reticularis at diestrous(Plate 1C), which become intense at proestrous (Plate 1D) witrh weak activity in the zona reticularis. However, the adrenocortical layers exhibited different intensities of peroxidase activity which appears to change with the reproductive cycle. The fasciculate and reticularis cells at metaestrous show high peroxidase activity (Plate 1B). No activity of peroxidase is observed without H_2O_2 in the system and cyanide exerted a powerful inhibitory effect.

Δ^{5} 3β- HYdroxysteroid dehydrogenase: A well developed cytoplasm of the zona fasciculata and reticularis show Δ^{5} 3β- Hydroxysteroid dehydrogenase at all the phases of the estrous cycle. Fine, granular,dense purple formazan deposits are seen in the zona glomerulosa and the outer layer of zona fasciculata of normal cycling rats (Plate 2 A& B and Plate 3 A,B & C) with diffuse activity in zona reticularis. The control sections without the substrate i.e. DHA show no activity thereby confirming the resence of this enzyme in these regions..

Cytochrome oxidase: The zona fasciculata and zona reticularis of the adrenal cortex show high activity of cytochrome oxidase at deestrous which reaches its zenith during proestrous (Plate 4A,B,C & D). The activity is low in these regions at estrous (Plate 5A & B). The control sections without the substratei.e. ά- naphthol show no activity, thereby confirming the presence of this enzyme in this region.

Lipids: A dense localization of sudanophilic lipids is seen in the zona fasciculata and reticularis at estrous and metaestrous (Plate 6A,B & C). A high lipid content is also seen in the zona glomerulosa (Plate 6 A & C). Decrease in lipid content is observed in zona fasciculata and reticularis at proestrous and moderate localization is seen at diestrous (Plate 7 A&B).

Acid and Alkaline phosphatases: In rat weak activity of acid phosphatase is seen at estrous in the adrenal cortex particularly in the zona fasciculate region (Plate 8A). There are significant differences in the localization of acid phosphatase during the reproductive cycle. With longer incubations the activity is detected in the cortical layers at diestrous and proestrous (Plate 8B & C). The entire adrenal cortex has high acid phosphatase activity at metaestrous which predominates in the fascicular zone and the reticular zone (Plate 9A& B).

In contrast to the above, a moderate activity of alkaline phosphatase is seen in the outer fascicularZone at metaestrous (Plate 10A). In the external two third of the fascicular zone and the external part ofthe reticular zone the activity of alkaline phosphatase varied with the cycle. A high activity of this enzyme is seen in the outer fascicular zone and in the reticular zone at diestrous (Plate 10B & C). Entire fascicular and reticular zones show intense activity at proestrous (Plate 1011A) which decreases at estrous except in the reticular zone where it increases markedly (Plate 11B). It appears that the activity of the alkaline phosphatase which becomes quite significant at diestrous and proestrous, while the adrenal lipids desrease, correlates well with the action of ACTH on the hypertrophied adrenal cortex to stimulate sex hormone synthesis in physiological condition.

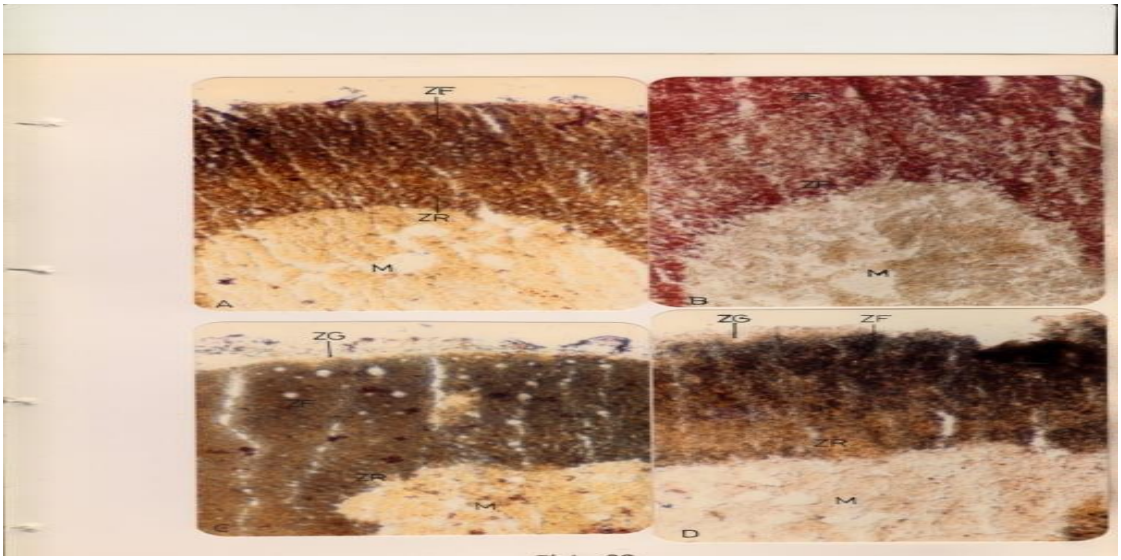

Plate 1

Localizatiion of peroxidase in the adrenal of rat during differen stages of reproductive cycle.

A. Showing low localization of peroxidase in the zona fasciculate (ZF) & the zona reticularis (ZR).

B. At metaestrous showin high peroxidase in the zona fasciculate (ZF) & zona reticularis (ZR) with no activity in the medulla (M).

C. At diestrous showing high activity of peroxidase in the zona fasciculata (ZF) & zona reticularis (ZR) with no activity in the medulla(M).

D. At proestrous showing intense activity of peroxidase in the zona fasciculate(ZF) & low activity in the zona reticularis(ZR).No activity is seen in the glomerulosa(ZG) & the medulla(M).

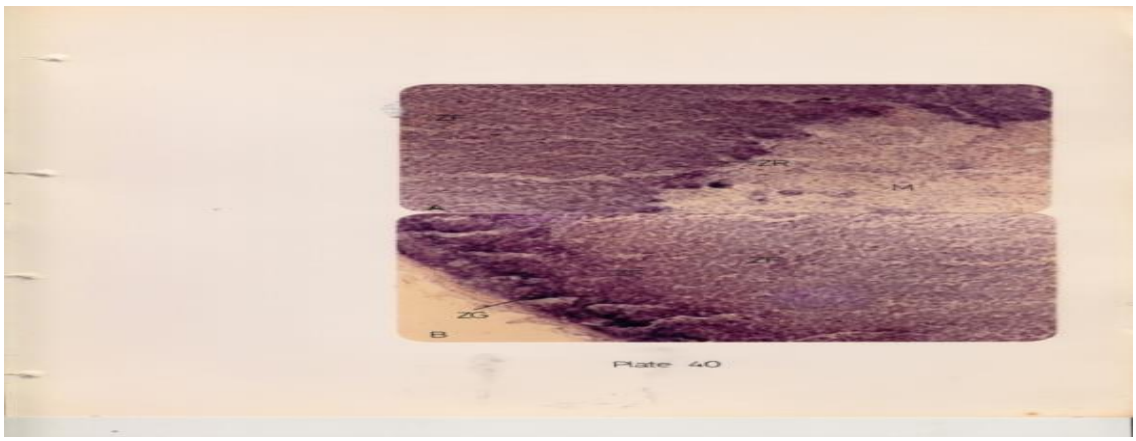

Plate 2

Localization of Δ^5-3β-Hydroxysteroid dehydrogenase in the adrenal of rat at proestrous of the reproductive cycle.

A. A section of adrenal showing diffuse localization of Δ^5- 3β- Hydroxysteroid dehydrogenase in the inner region of fasciculate (ZF) & zona reticularis (ZR). No activity is seen in theMedulla(M).

B. Section of adrenal showing high activity of Δ^5- 3β-hydroxysteroid dehydrogenase in the zona glomerulosa(ZG) and the outer region of zona fasciculate(ZF) while diffuse localization is seen in the inner region of (ZF).

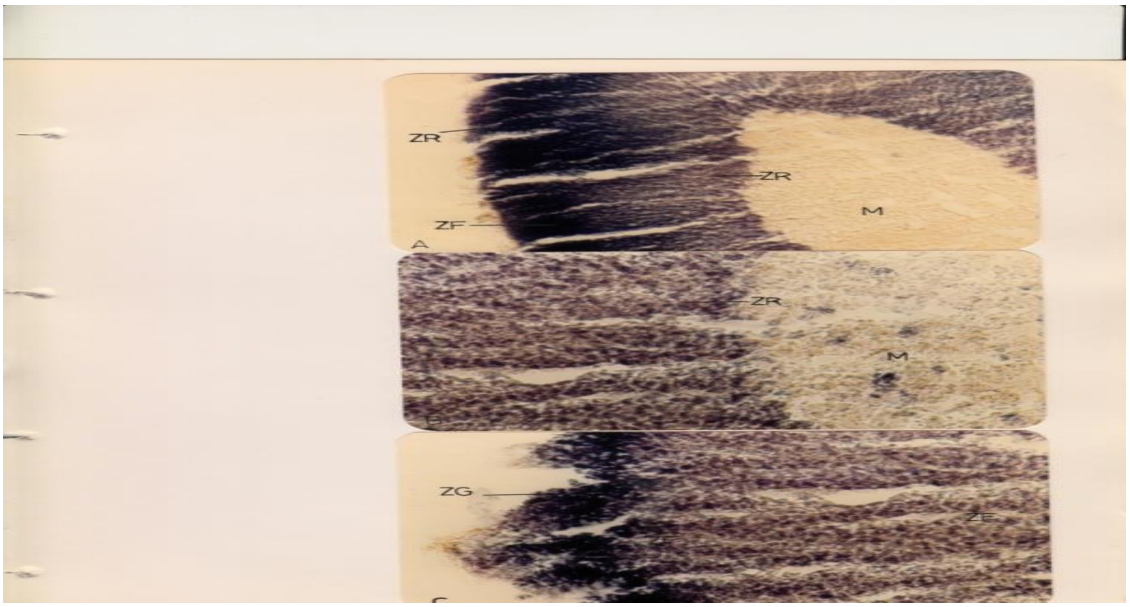

Plate 3

Showing localization of Δ^5-3β-Hydroxysteroid dehydrogenase in the adrenal of rat at diestrous & estrous of the reproductive cycle

A. Section showing diffuse localization of ^{5}-3β- Hydroxysteroid dehydrogenase in the inner region of zona fasciculate (ZF) & zona reticularis (ZR). No activity seen in the medulla (M). High activity seen in the zona glomerulosa(ZG).

B. section at estrous under high power showing high activity of ^{5}- 3β- hydroxyl- Steroid dehydrogenase in the zona reticularis (ZR). No activity seen in medulla (M).

C. A section under high power showing high activity in the zona fasciculate (ZF) while intense localization is seen in the zona glomerulosa (ZG).

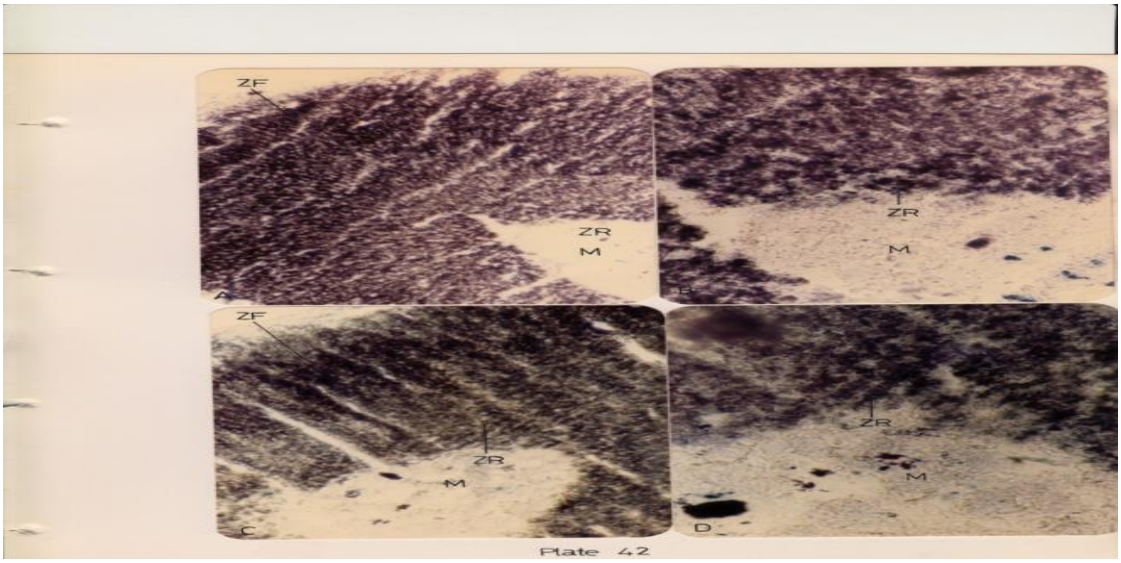

Plate 4

Localization of cytochrome oxidase in the adrenal of rat at diestrou and proestrous of the reproductive cycle

A. Adrenal of rat at diestrous showing high activity of cytochrome oxidase In the zona fasciculate (ZF) & zona reticularis (ZR), while no activity seen in the medulla (M).

B. A portion under high power showing intense localization of cytochrome oxidase in the zona reticularis (ZR).

C. Adrenal of rat at proestrous showing intense activity of cytochrome Oxidase in the zona fasciculate (ZF) and zona reticularis (ZR) with no activity. in the medulla (M).

D. A section under high power showing intense localization in the zona reticularis (ZR)

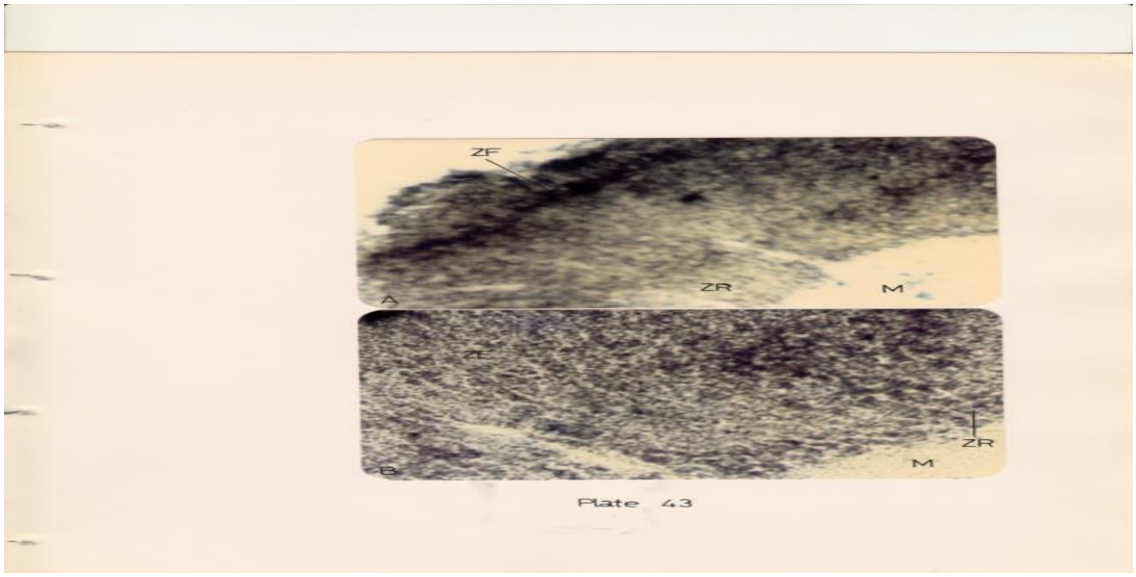

Plate 5

Showing localization of cytochrome oxidase in the adrenal of rat at estrous of the reproductive cycle.

A. Adrenal of rat showing low cytochrome oxidase activity in the outer region of zona fasciculate (ZF) while diffuse is seen in zona reticularis(ZR). No activity is seen in the medulla (M).

B. A portion under high power showing diffuse localization of cytochrome oxidase in the inner region of zona fasciculate (ZF) & in zona reticularis (ZR).

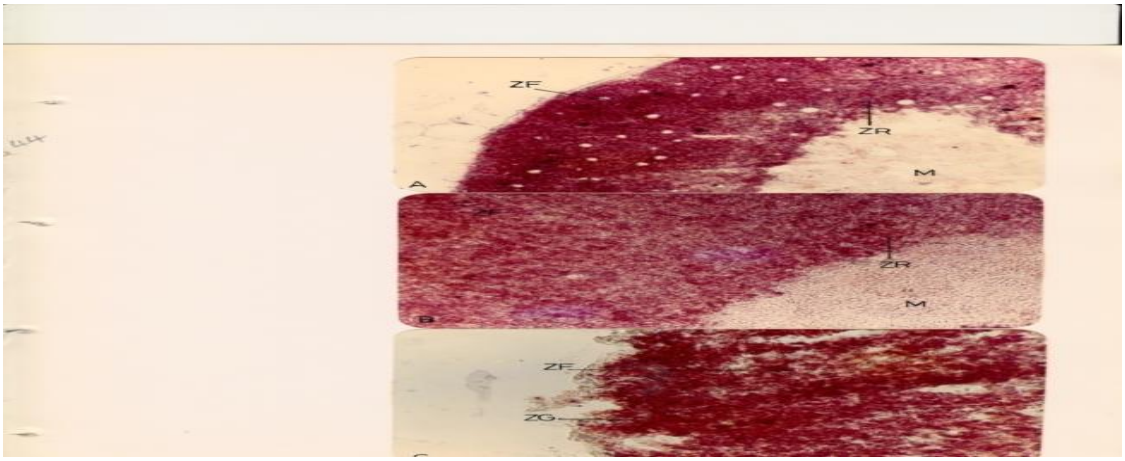

Plate 6

Showing localization of lipids in the adrenal of rat during during different stages of reproductive cycle

A. Aderenal of rat at estrous showing abundant sudanophilic lipids in the zona fasciculate (ZF) & zona reticularis (ZR). No activity is seen in the medulla (M).

B. A portion of rat adrenal at metaestrous under high power showing lipids in the zona fasciculate (ZF) and zona reticularis (ZR).

C. A portion of rat adrenal under high power showing abundant lipids in the zona fasciculate (ZF) & zona glomerulosa(ZG).

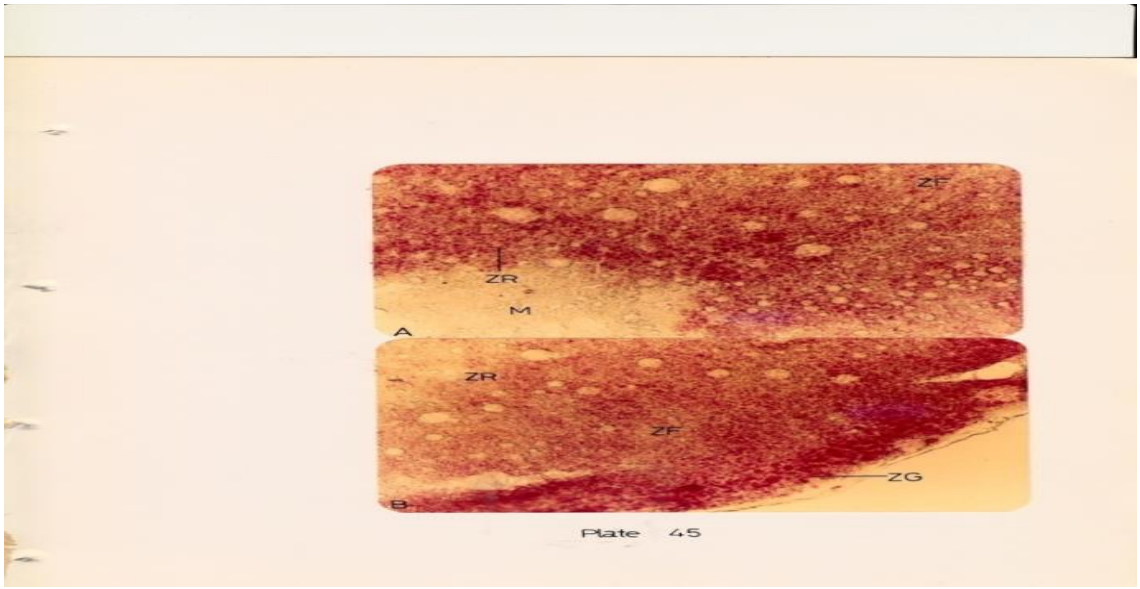

Plate 7

Localization of lipids in the adrenal of rat during different stages of reproductive cycle

A. A portion of rat adrenal at proestrous showing diffuse localization of lipids in the zona fasciculata (ZF) & zona reticularis (ZR). No activity is seen in the medulla (M).

B. Adrenal of rat at diestrous showing sudanophilic lipid droplets in the zona glomerulosa (ZG)and zona fasciculate (ZF) while weak localization is seen in the zona reticularis (ZR).

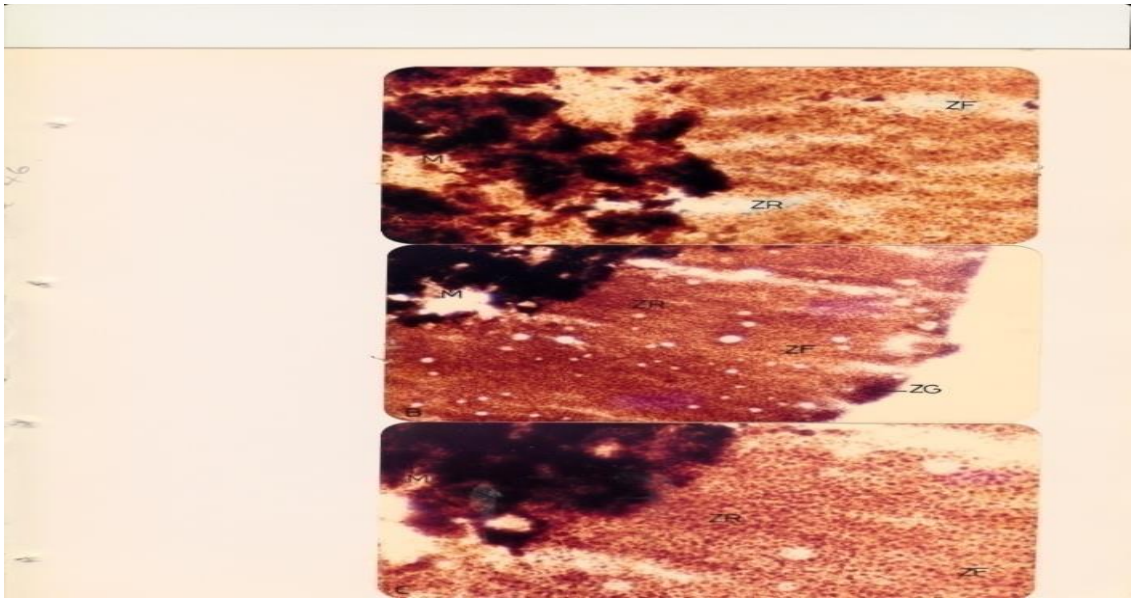

Plate 8

Showing localization of acid phosphatase in the adrenal of rat duringdifferent stages of reproductive cycle.

A. A portion of rat adrenal at estrous showing weak activity in the zona fasciculata (ZF) while diffuse localization is seen in the zona reticularis (ZR). A very high activity is seen in the medulla (M).

B. A rat adrenal at diestrous showing uniform localization of acid Phosphatase inlata the zona fasciculate (ZF) and zona reticularis (ZR). A very high activity is seen in the medulla (M)).

C. A portion of rat adrenal at proestrous showing uniform locali- zation in the zona fasciculate (ZF) & zona reticularis (ZR) with high activity in the the medulla (M).

Plate 9

Localization of acid phosphatase in the adrenal of rat at metaestrous of the reproductive cycle

A. portion of rat adrenal under high power showing high activity in the zona fasciculate (ZF) and zona reticularis (ZR). A high activity is seen in the medulla (M).

B. A portion showing high activity in the zona glomerulosa (ZG) and zona fasciculate (ZF)

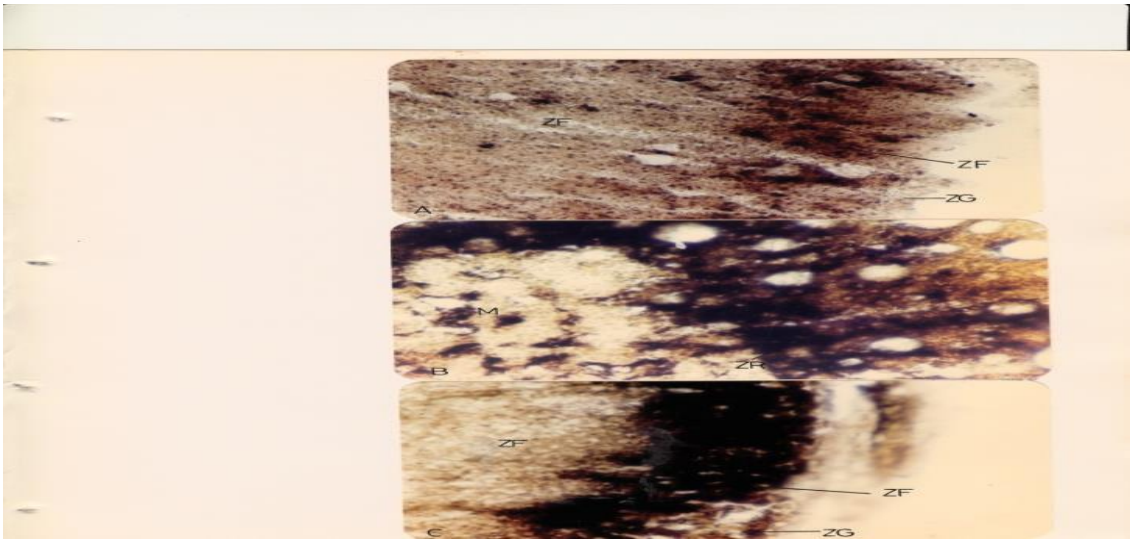

Plate 10

Showing localization of alkaline phosphatase in the adrenal of rat during different stages of the reproductive cycle.

A. A portion of rat adrenal at adrenal at metaestrous showing moderate Activity in the outer region of zona fsciculata (ZF).

B. A portion of rat adrenal diestrous showing intense localization in the zona reticularis (ZR), while no activity is seen in the medulla (M).

C. A section of rat adrenal at diestrous showing intense activity in outer region of zona fasciculate (ZF) with diffuse localization in the inner region.

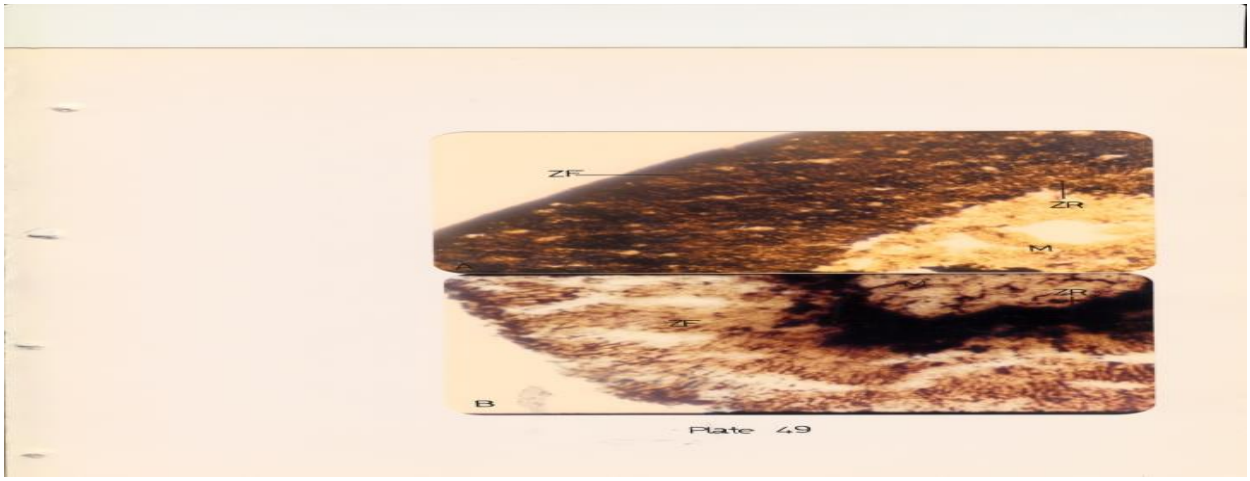

Plate 11

Showing localization of alkaline phosphatase in the adrenal of rat during different stages of reproductive cycle.

A. Adrenal of rat at proestrous showing intense localization in the zona fasciculata (ZF) and zona reticularis (ZR) with no activity in the medulla (M).

B. Adrenal of rat at estrous showing very high activity in the zona reticularis (ZR) with low activity in the zona fasciculate (ZF).

IV. DISCUSSION

Peroxidase is present in the inner layer of a drenocortical Cells 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 compartments. 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 . Presence of high peroxidase activity in 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 acts 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 blood Flow 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 these sites 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 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.

V. ACKNOWLEDGEMENT

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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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