

Histochemical Studies of Enzymes in the Adrenal Gland of Rat & Rabbit during Pregnancy

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Abstract: Administration of ACTH stimulates adrenal secretion of progesterone as well as corticosterone (Resko, 19691; Feder et al., 1969; Feder et al., 1971; Piva et al., 1973). Progesterone is both an obligatory intra-adrenal substrate for corticosterone production and a steroid essential for maintenance of pregnancy. Thus, the regulation of adrenal steroidogenesis during pregnancy has two potentially important aspects: i.) Maintenance of optimal blood levels of corticosterone and ii.) Contributing significant amounts of progesterone to the total maternal pool. Since the extended luteotrophic function of ovary in rat & mice during pregnancy is related to the Peroxidase-Ascorbate system (Agrawal, P. & Laloraya, M.M. 1979). It appears likely that synthesis of progesterone under the action of ACTH during pregnancy may be controlled by a similar mechanism as reported for LH in the ovary, thus causing increased synthesis and secretion of the Progesterone and corticosteroids from the adrenal gland.

1. INTRODUCTION

The various in vivo and in vitro studies have demonstrated that the ovary and adrenal possess the side-chain cleaving system to convert C₂₇ cholesterol to pregnenolone which are mainly a C₂₂-C₂₀ 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 adrenal which is closely interlinked in reproductive functioning of different groups of animals remains largely unknown. Also the hormone regulation and the enzymic mechanism which lead to the rapid formation and secretion of hormone namely progesterone and corticosteroids in the adrenal gland is largely unknown.

ACTH has a major role in the synthesis of progesterone which is known to be a precursor of several steroid hormones including androgens, estrogens and corticoids (Gorbman and Bern, 1974). A preovulatory surge of progesterone in the systemic plasma of adrenal origin preceeding the display of behavioural estrous in rodents has been reported (Feder et al., 1968).ACTH is also known to cause depletion of adrenal ascorbate and cholesterol in the hypophysectomized rat (Tyslowitz, 1943; Sayer et al., 1946) which is shown to occur within minutes of ACTH injection and to exhibit a characteristic time sequence.

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2. MATERIAL & METHODS

Colony-bred albino rats (Wistar Strain) of our departmental colony maintained on a regimen of 12hrs.light/12 hrs.dark in a temperature controlled room ($25^{\circ} - 30^{\circ} \text{C}$) were used in this study. They received food & water ad libitum. 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 estrous cycle. The adrenal of sexually mature rat were used. Pregnancy, which lasted 21 days in rats, was induced by mating functional females with proven males on the day of vaginal cornification, which was designated day 0. The day of conception was ascertained by examining vaginal smears daily; the observed presence of sperms in a smear being taken to indicate the first day of pregnancy. The pregnant females were sacrificed at various stages of pregnancy by cervical dislocation and dissected. The adrenal were stored at -20°C and were later subjected to various histochemical analyses.

Colony bred mature female white rabbits of our departmental colony were caged individually in a controlled environment with light-dark cycle of 14:10 hours. Water and food were supplied ad libitum. They were mated twice with different bucks of proven fertility followed by i.v. injection of 100 i.u. of human chorionic gonadotropin (CG-5, Sigma Chemicals co. USA) to induce ovulation and were designated as pregnant rabbits. The pregnant females were anesthetized by i.v. injection of Sodium pentobarbitone at various stages of pregnancy. The dissected tissues were stored at -20°C . The adrenals were subjected to histochemical studies.

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 Benzidine 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. $\Delta^5\beta$ - 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) was followed.

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

7. Ascorbic Acid: Histochemical localization of ascorbic acid brought about by the method of Chenoy (1969b).

3. RESULTS

Peroxidase:

Peroxidase is observed to be detectable during early gestation period in rat & rabbit. Low activity of peroxidase is observed in zona glomerulosa, zona fasciculate and zona Reticularis (Plate 1A & B). A diffuse activity of this enzyme is seen in the blood vessels of zona reticularis during the first day of pregnancy in rabbit (Plate 2A & B). The activity of peroxidase increases on days 4-7 of pregnancy in the zona fasciculate of rat & rabbit while low activity is seen in the zona reticularis (Plate 2A, B, C & D and Plate 3A). During the later periods of gestation, the activity of peroxidase

decreases gradually in the zona fasciculata and is minimum on day 15 of pregnancy (Plate 3B). Thereafter, the activity of this enzyme remains constant showing very diffused localization in the three zones of the cortex. No activity was observed without H₂O₂ in the system and cyanide exerted a powerful inhibitory effect (Plate 3 C & D)

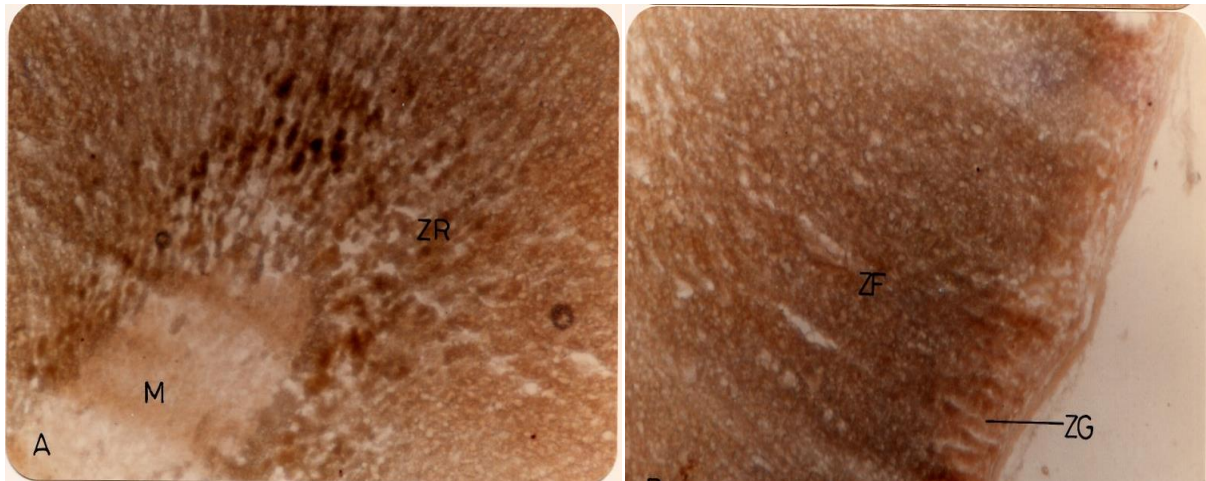


Plate 1A&B: Showing localization of peroxidase in the adrenal of pregnant rabbit at day 3 of pregnancy.

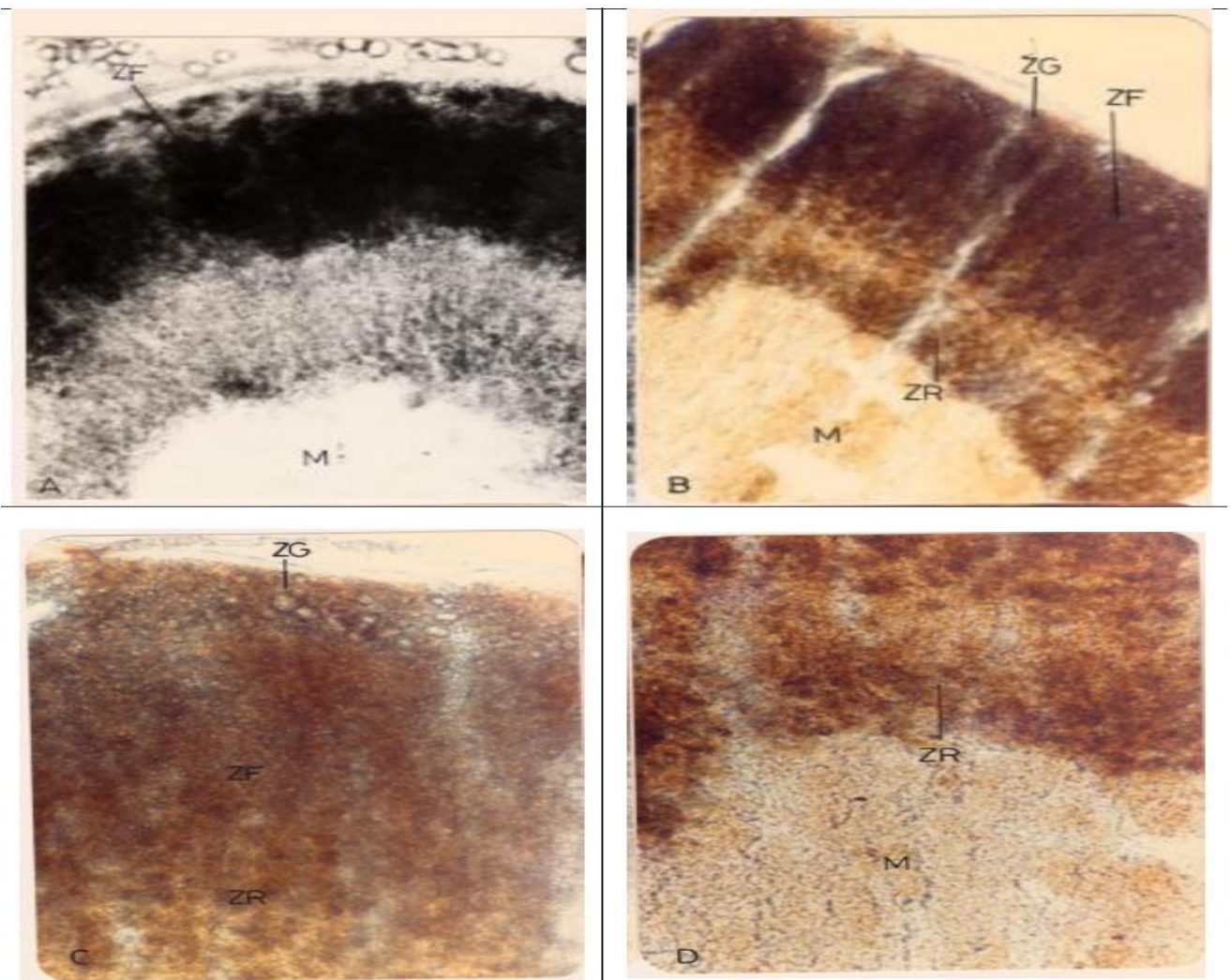


Plate.2: A, B,C&D: Showing localization of peroxidase in the adrenal of rat at day 6 of pregnancy.

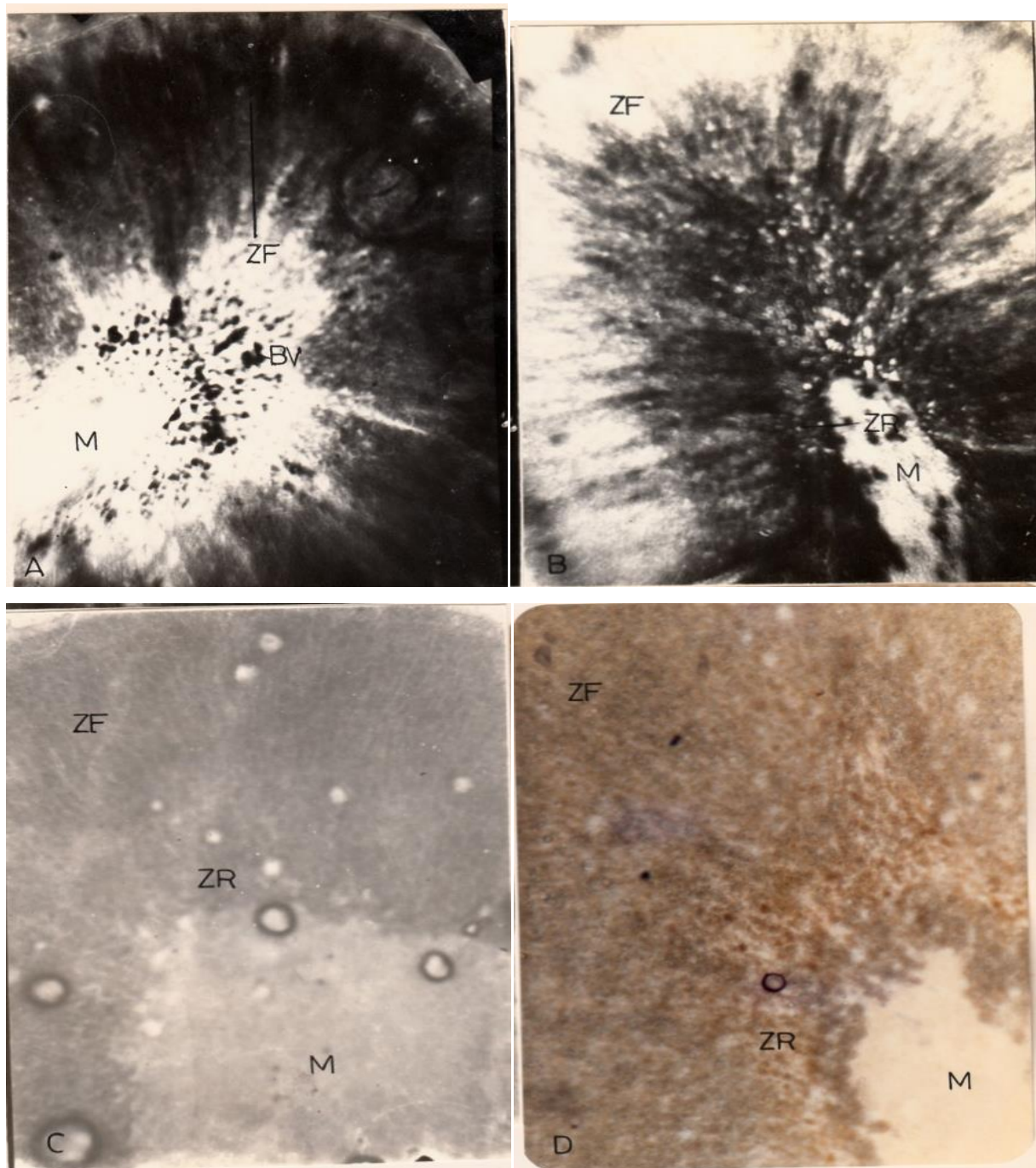


Plate.3: A&B: Showing localization of peroxidase in the adrenal of pregnant rat & rabbit at different days of pregnancy. C&D: Cyanide treated Control sections of adrenal of rat & rabbit showing no activity of peroxidase.

Hydroxysteroid dehydrogenase

The enzyme activity is, in general, very strong in the mammals and shows significant Species differences. Under similar conditions it has been shown to be absent in the mouse Adrenal, weak in rabbit & very strong in pregnant mouse, cow sheep and dog (Rubin et al., 1963). In rat and rabbit steroid dehydrogenase is mainly localized in the outer part of the zona fasciculata and average activity is seen in the zona reticularis during early days of pregnancy (Plate 4A, B & C). Higher activity of Δ^5 -3 β -Hydroxysteroid dehydrogenase is maintained in the zona fasciculata throughout the early gestation period in rat & rabbit (Plate 5A, B & C). The activity of Δ^5 -3 β -Hydroxysteroid dehydrogenase decreases in the zona fasciculata & zona reticularis of rat & rabbit during later days of pregnancy (Plate 6A & B).

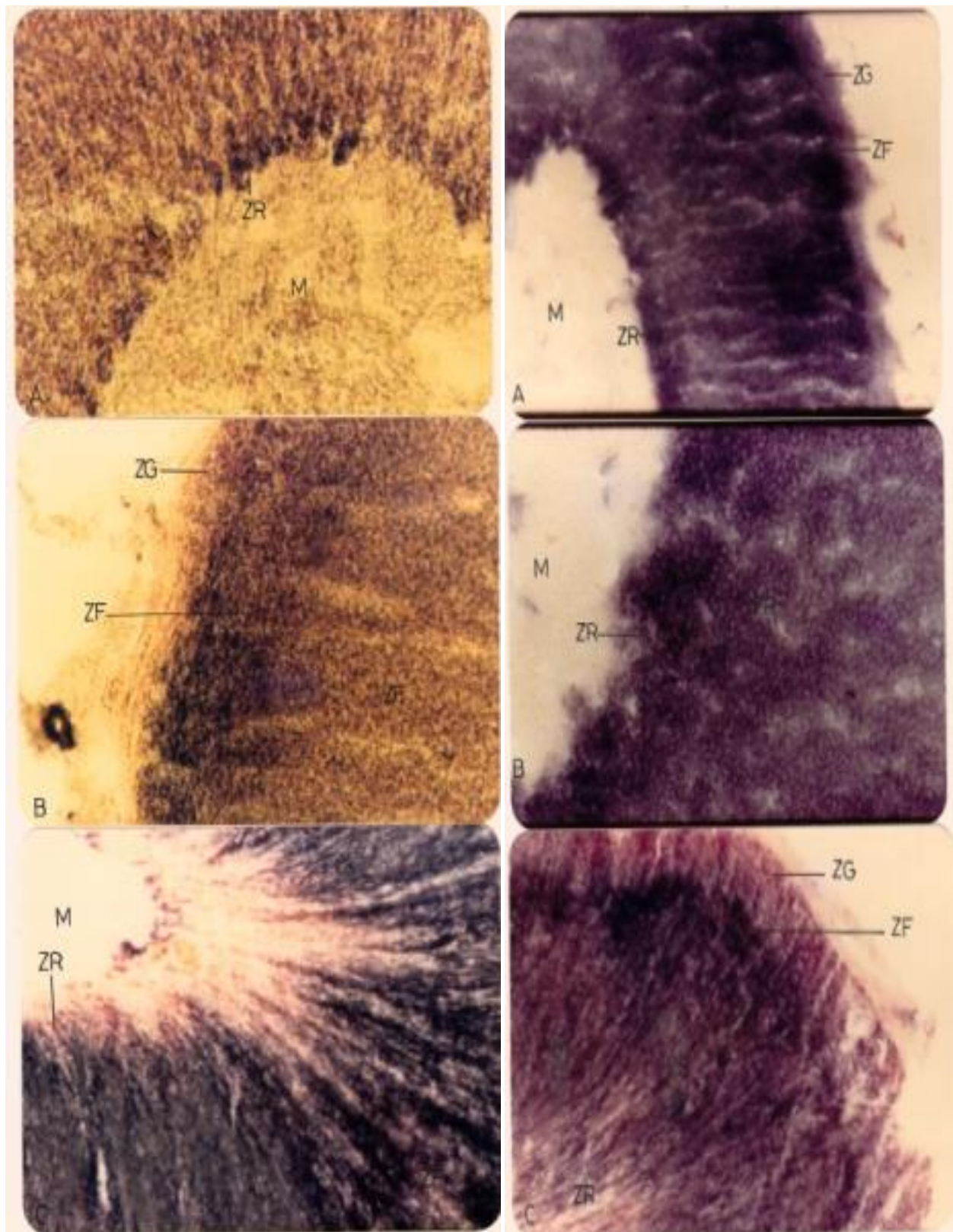


Plate.4: A, B&C: Showing localization of Δ^5 -3 β -Hydroxy Steroid dehydrogenase in the pregnant rat & rabbit adrenal gland at day 5 of pregnancy

Plate 5A, B&C: Showing localization of Δ^5 -3 β -Hydroxysteroid dehydrogenase in the pregnant rat & rabbit adrenal gland at day 12 of pregnancy.

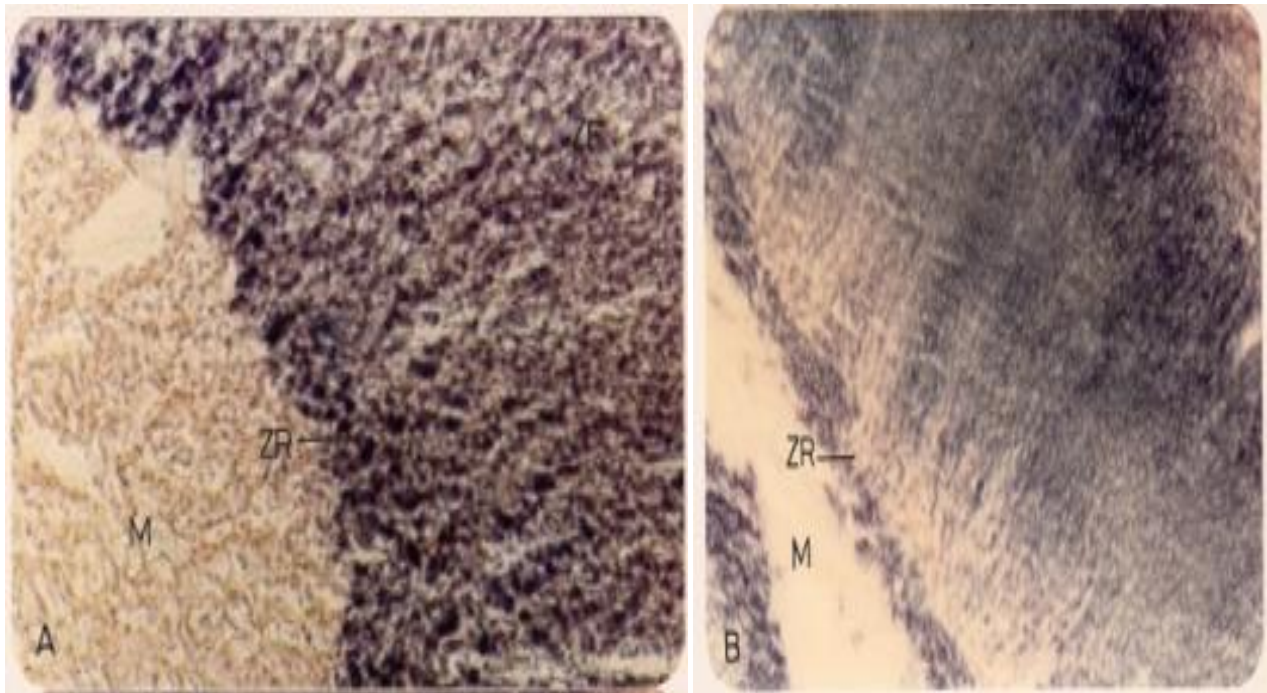


Plate 6A&B: Showing localization of Δ^5 -3 β -Hydroxysteroid dehydrogenase in the pregnant rat & rabbit adrenal gland at day 18 of pregnancy.

Cytochrome oxidase

A positive Cytochrome oxidase activity is seen in the adrenals of rat and rabbit during pregnancy. The Enzymes activity is low in the zona fasciculata and reticularis layers at days 1-4 of pregnancy (Plate 7A & B). A high activity of Cytochrome oxidase is seen in the fascicular zone from days 6-14 of pregnancy In rat and rabbit (Plate 8A,B & C) and decreases gradually during the later period of gestation (Plate 9A,B & C). The control sections without the substrate i.e. α -naphthol show no activity thereby confirming the presence of the enzyme in these regions (Plate 9D).

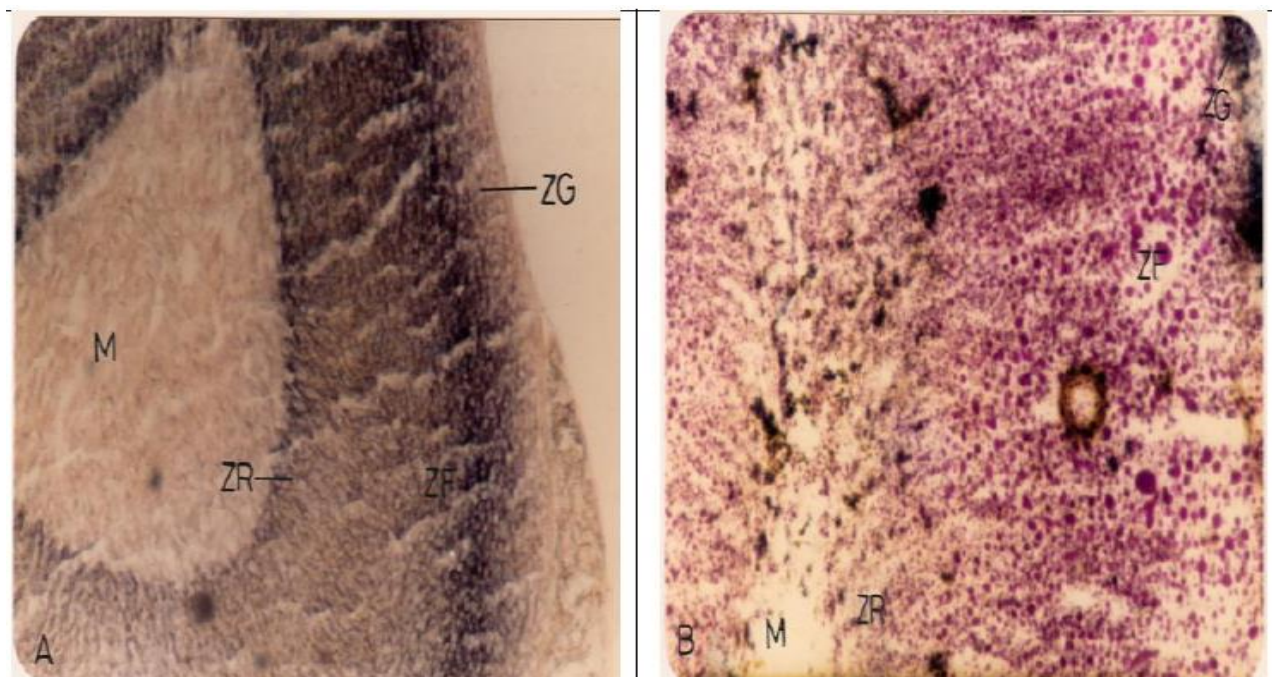


Plate.7: A&B: Showing localization of Cytochrome oxidase in the pregnant rat & rabbit at day 3 of pregnancy.

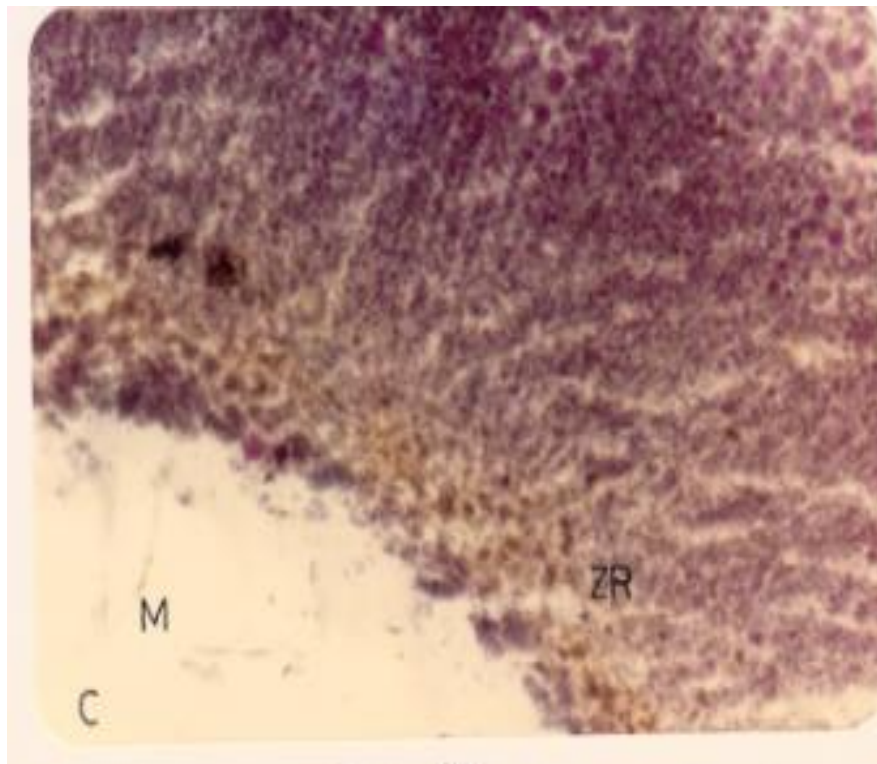
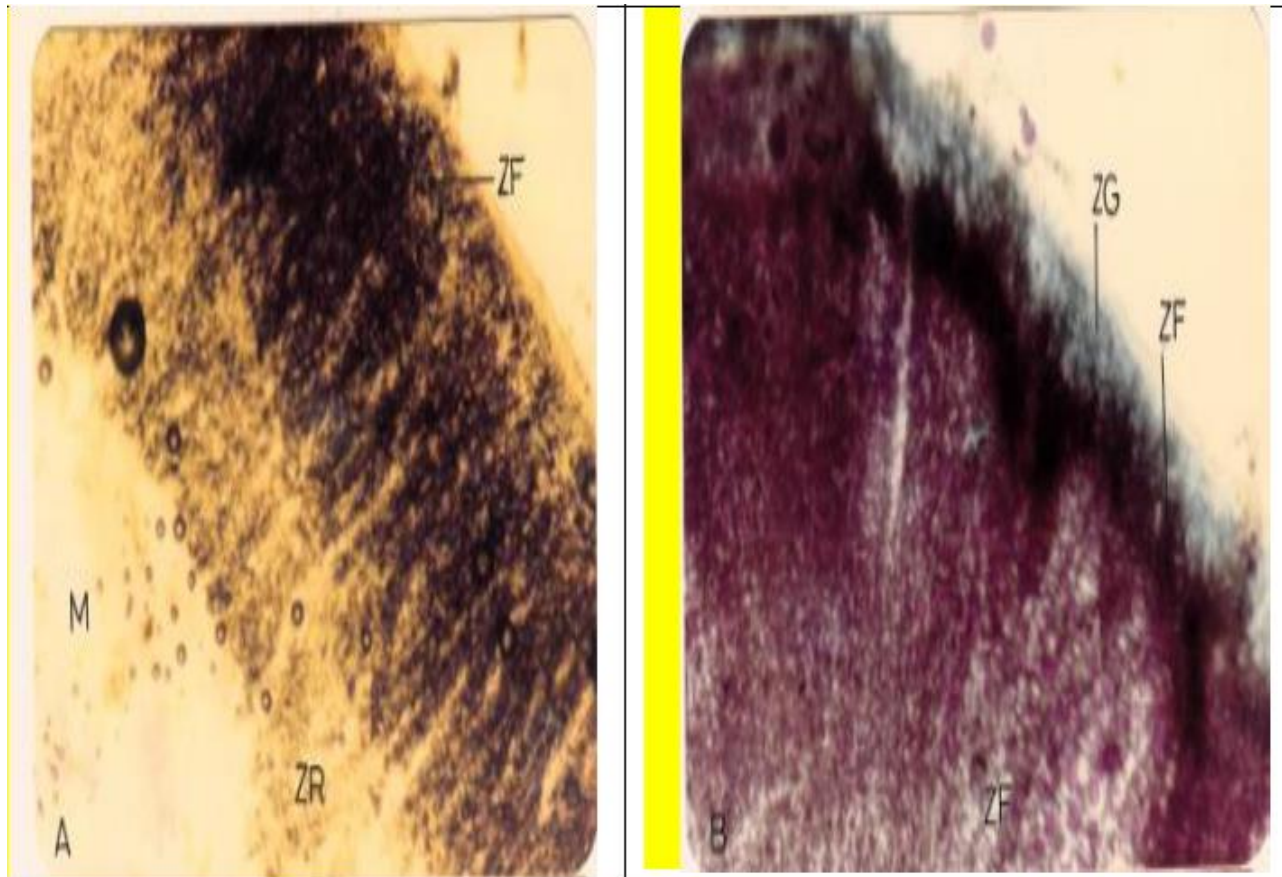


Plate.8: A,B&C: Showing localization of Cytochrome oxidase in the adrenal of rat pregnant rat at day 10 & rabbit at day 14 of pregnancy.

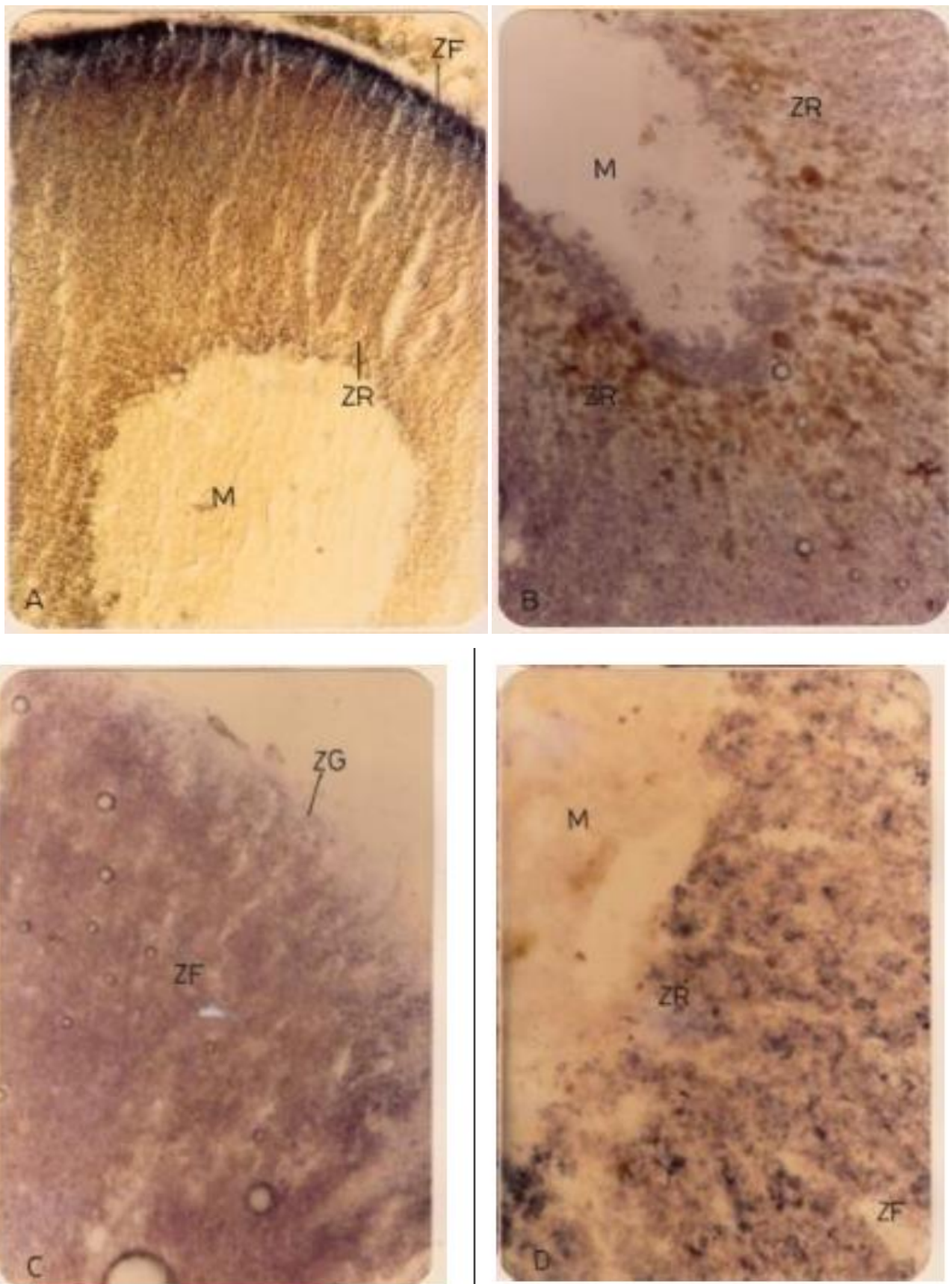


Plate 9: A,B,C&D: Showing localization of Cytochrome oxidase in the adrenal of rat & rabbit at day 18 of pregnancy.

Alkaline phosphatase

A number of authors have noted the difference in localization of the adrenal alkaline phosphatase Activity depending on the species examined (Nicander, 1952). In our studies the entire adrenal cortex

of rat and rabbit has a histochemically detectable alkaline phosphatase activity during pregnancy, which predominates in the zona glomerulosa, outer zona fasciculata and zona reticularis (Plate 10A,B,C & D). A high activity of alkaline phosphatase is seen in the zona reticularis on day 16 of pregnancy while it is low in the zona glomerulosa in rat (Plate 11A & B) and rabbit (Plate 11 C). Elftman (1947) has shown that female adrenal cortex is rich in alkaline phosphatase activity mainly in the reticular zone which correlates very well with our histochemical observations. No activity is seen in the control sections i.e., minus the substrate, Sodium- β - glycerophosphate, thereby confirming the presence of this enzyme to these regions (Plate 12 A & B).

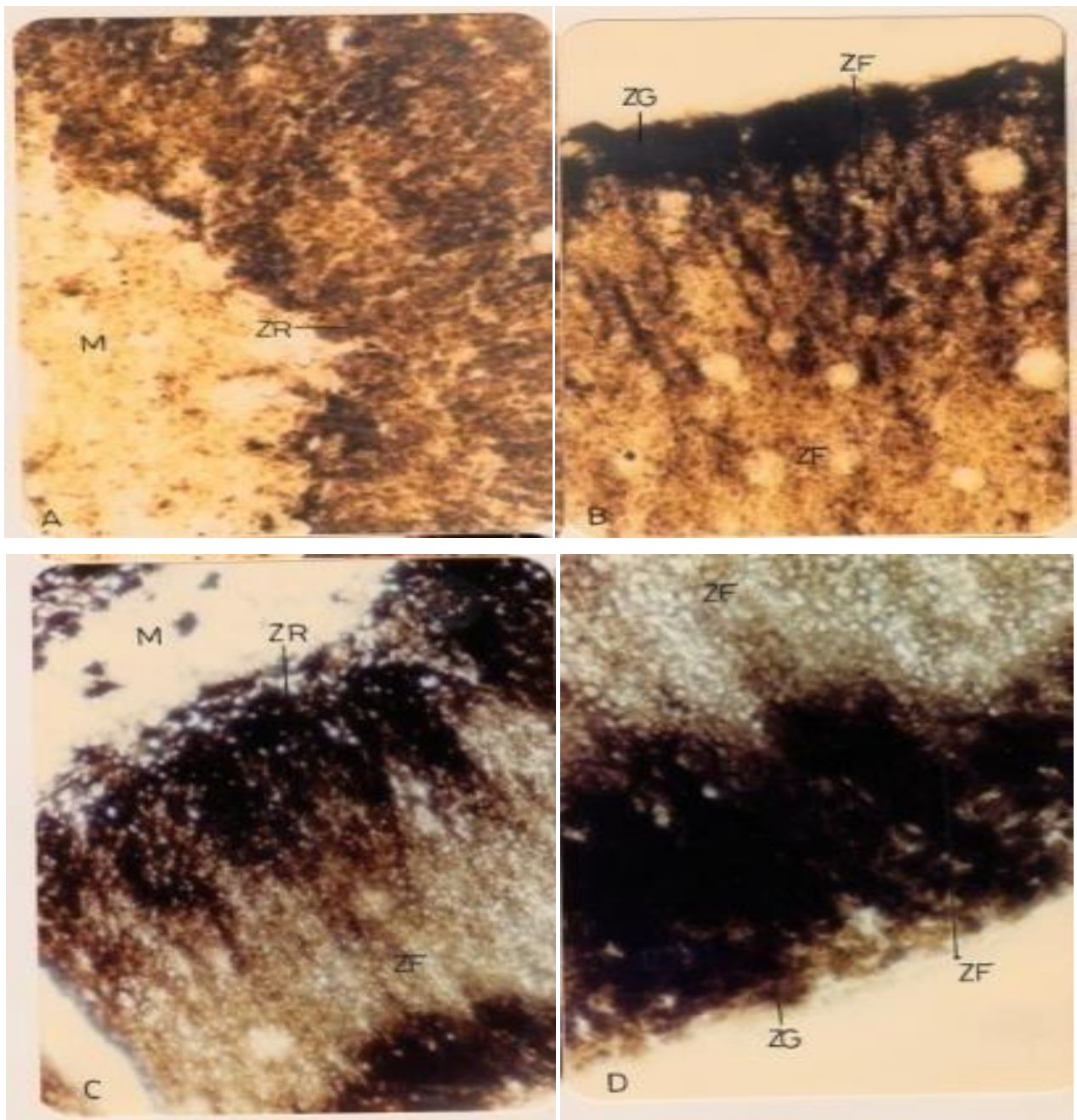


Plate.1: 0A,B,C&D: Showing localization of alkaline phosphatase in the adrenal of pregnant rat and rabbit at day 6 of pregnancy.

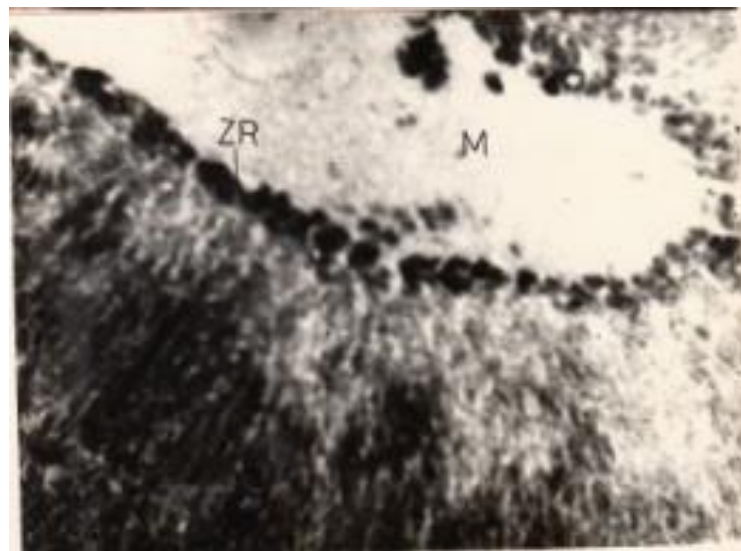
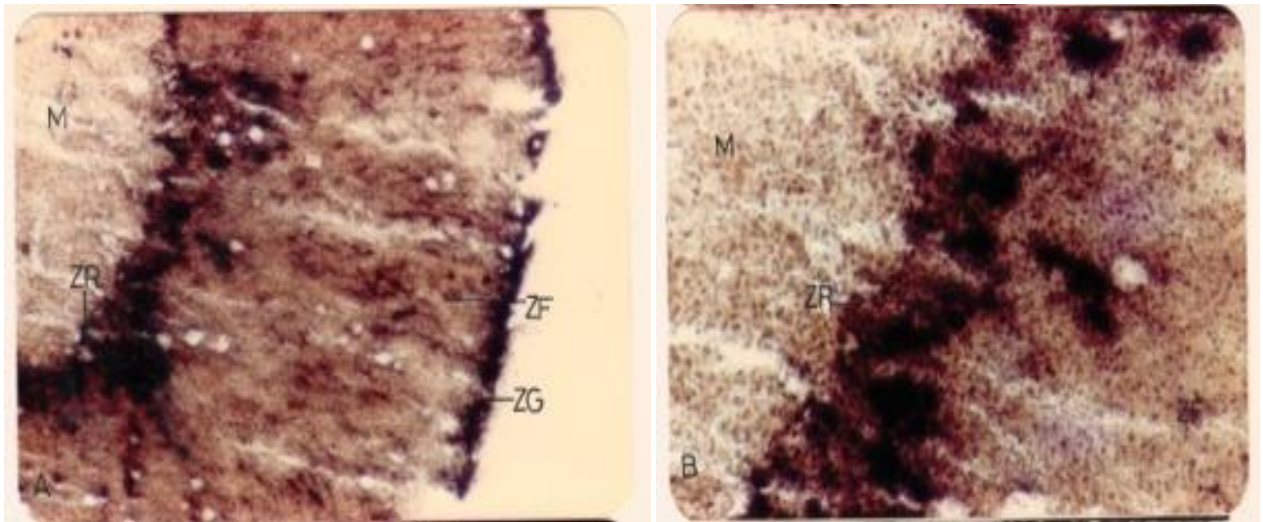


Plate 11 A,B&C: Showing localization of alkaline phosphatase in the adrenal of pregnant rat and rabbit day 16 of pregnancy.

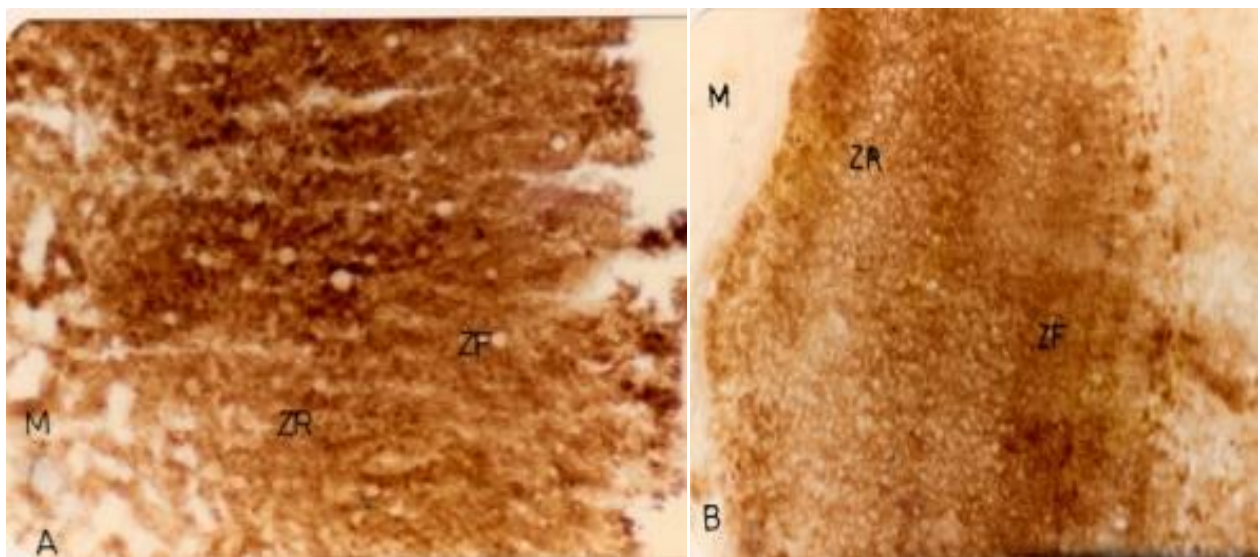


Plate 12A&B: Control sections of rat & rabbit adrenal minus the substrate showing no activity of Alkaline phosphatase at day 6 of pregnancy.

Acid phosphates:

In mammals, the histochemically detectable acid phosphatase activity is more uniformly distributed than the alkaline phosphatase. The acid phosphatase activity is high in the fascicular zone and in the reticular zone on day 4 of pregnancy in rat and rabbit (Plate 13A, B & C). A uniformly distributed activity of acid phosphatase is seen in the three layers of the cortex on day 10-12 of pregnancy (Plate 14A,B & C) The glomerular zone is richer than the rest of the cortex in rabbit (Plate 14C). In rat it is clearly less rich than the rest of the cortex (Plate 14A). In rabbit, the enzyme activity predominates in the internal zone (Plate 14 B). In rat the entire cortex is poor in acid phosphatase and contrasts with the adrenal medulla which has an extremely strong activity(Plate14A). No activity is seen in the control sections i.e., minus substrate Sodium- β -glycerophosphate (Plate 15A & B), thereby confirming the presence of this enzyme in these regions

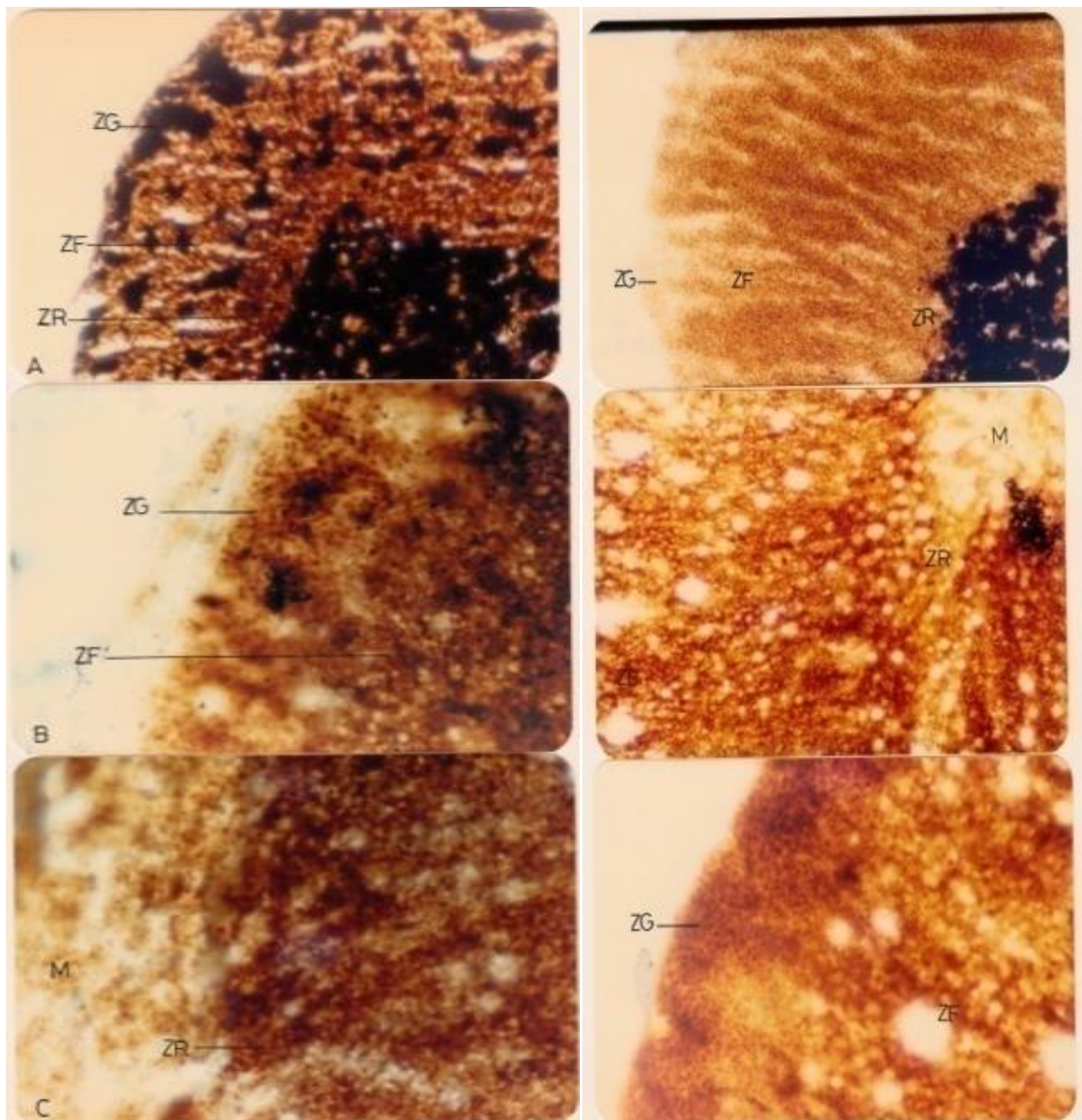


Plate 13 A,B&C: Showing localization of acid phosphatase in the pregnant rat & rabbit adrenal at day 4 of pregnancy.

Plate 14 A,B&C: Showing localization of acid phosphatase in the pregnant rat & rabbit adrenal at day 10 of pregnancy.

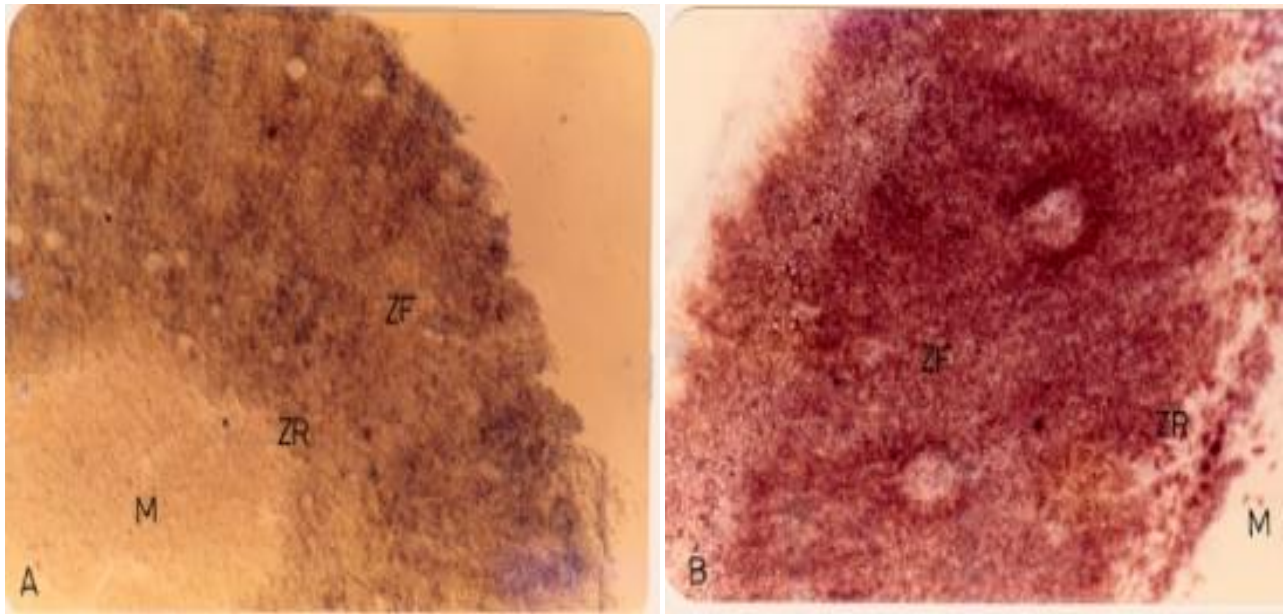
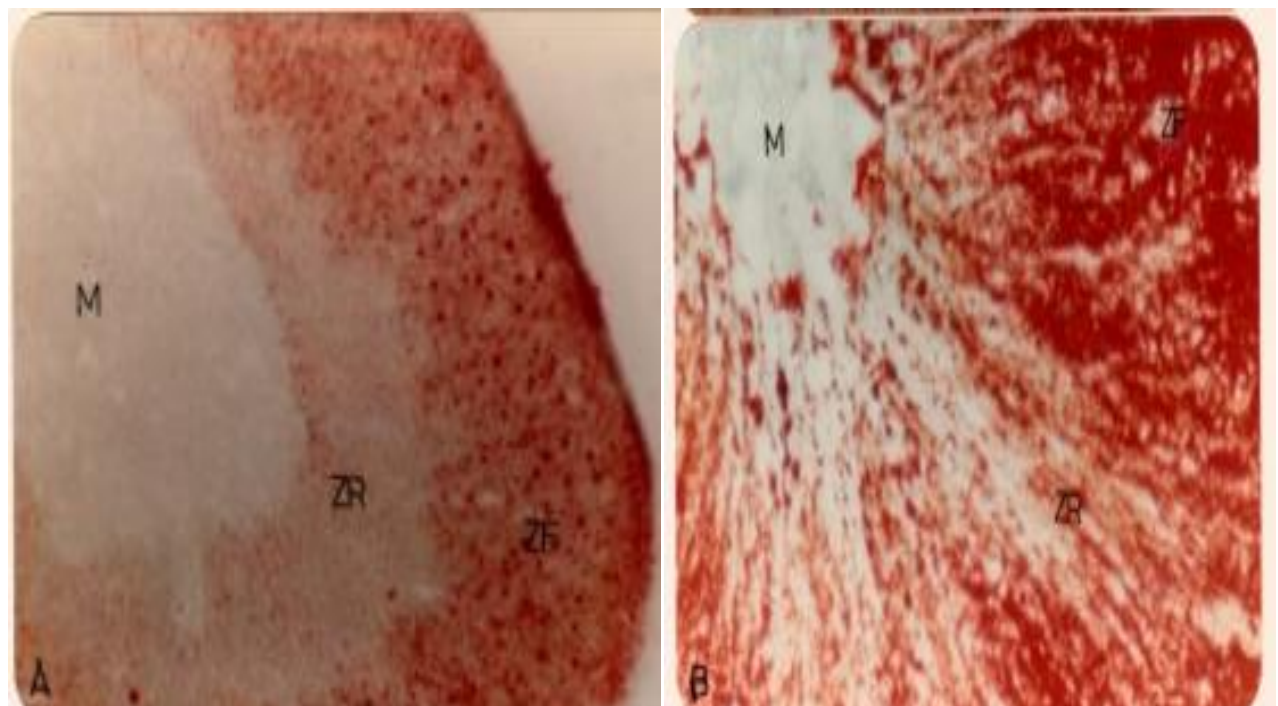


Plate 15 A&B: control sections of rat& rabbit adrenals the substrate showing no activity of acid phosphatase in the cortex (C) and medulla (M).

Lipids

The fat laden fasciculate cells are filled with sudanophilic lipids on days 4 and 5 of pregnancy, while sparsely scattered lipid bodies are seen in the zona reticularis of the rat and rabbit adrenals (Plate 16A, B & C). The dense localization of lipids is seen in the zona fasciculata in rabbit on days 8-16 of pregnancy (Plate 17A & B) while on days 10 and 16 in rat (Plate 17C). In the later days of pregnancy the fasciculata and reticularis layers show diffuse lipid droplets in rabbit (Plate 17D), while no change is seen in rat. Cholesterol has been shown to be the precursor in the steroid biosynthesis. The presence of lipids demonstrated histochemically in the cells of zona fasciculata provide sufficient amounts of cholesterol for use as a substrate for steroid synthesis.



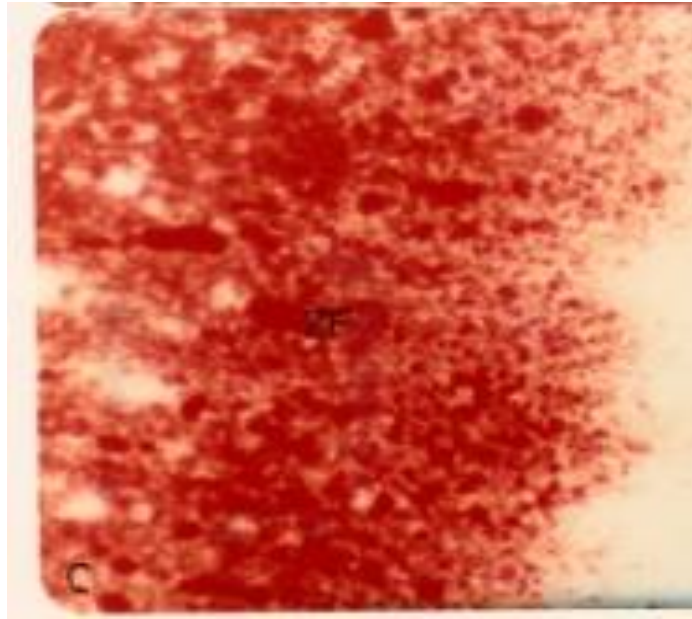


Plate 16 A,B&C: Showing localization of lipids in the adrenal of pregnant rat at day 4 of pregnancy.

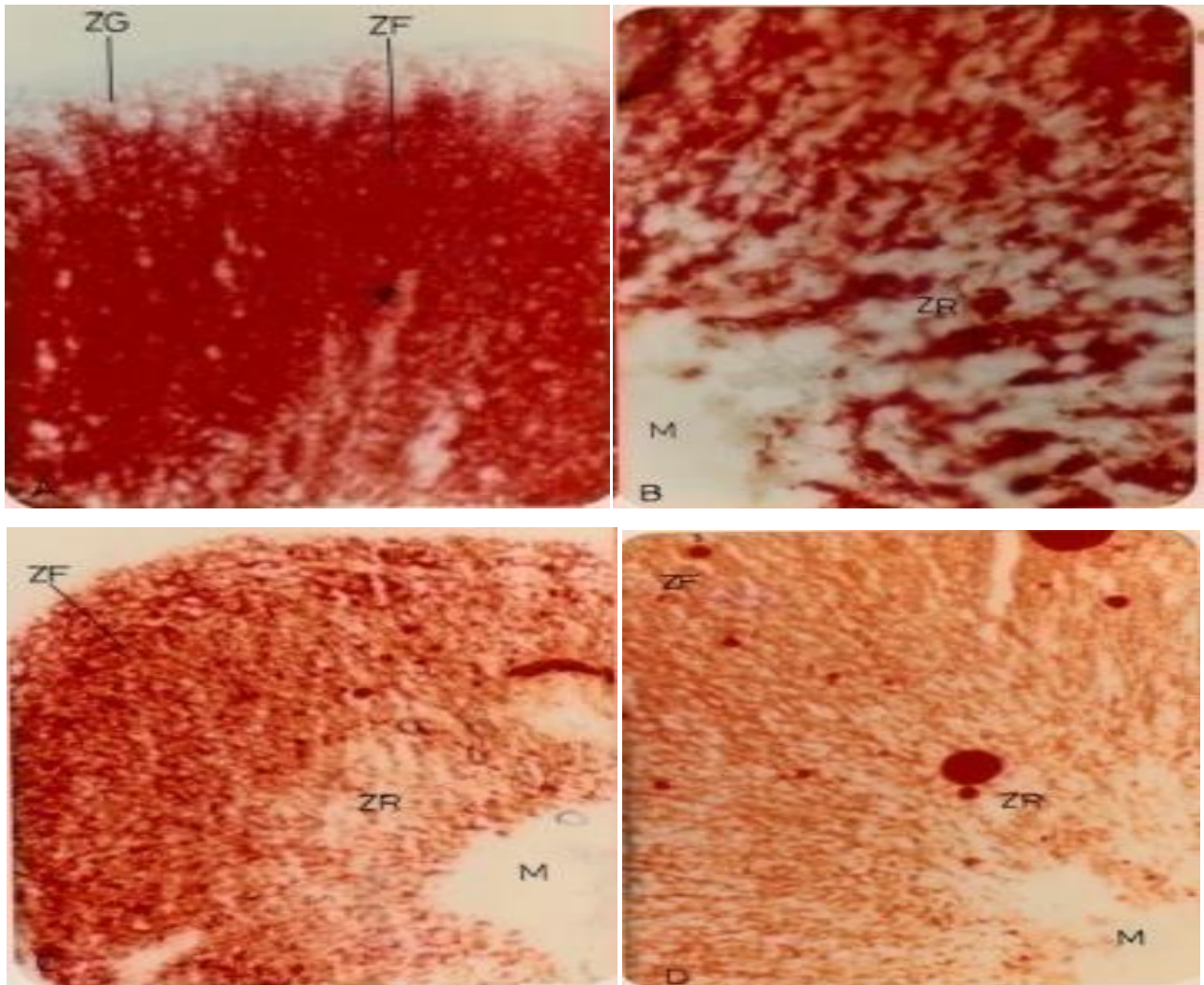


Plate 17A,B,C&D: Showing localization of lipids in the adrenal of pregnant rat & rabbit at day 11 of pregnancy.

Ascorbic acid:

The inner region of zona fasciculata and zona reticularis of the adrenal cortex in rat and rabbit is darkly stained during the first 4 days of pregnancy (Plate 18A, B, C & D and Plate 19A, B, C & D) indicating the presence of ascorbic acid. The polyhedral columnar cells contain a fine deposition of silver granules. The reticular cells of the hypertrophied cortex show deposition of silver granules in the outer region while the zona glomerulosa cells show black crystals indicating high concentration of ascorbic acid(Plate 20A&B). The chromaffin cells show brown diffuse localization which is not a characteristic of ascorbic acid. From days 5-11 of pregnancy the ascorbate content is very low. The zona fasciculata and reticularis layers show diffused & scattered deposits of silver granules (Plate 21A, B & C and Plate 22A, B & C) in rat and rabbit suggesting very low levels of ascorbate in the adrenal cortex. The high staining reaction in zona fasciculata is observed from day 13 onwards while moderate reaction is seen in zona reticularis (Plate 22A, B, C & D) which is not seen in the later days of pregnancy that is before parturition in rat and rabbit. Thus the histochemical studies clearly show that the zona fasciculata is characterized by the presence of high peroxidase, $\Delta^5 - 3\beta$ -Hydroxysteroid dehydrogenase, acid phosphatase and alkaline phosphatase activity while moderate to low activity is seen in the reticular zone of the adrenals of rat and rabbit.

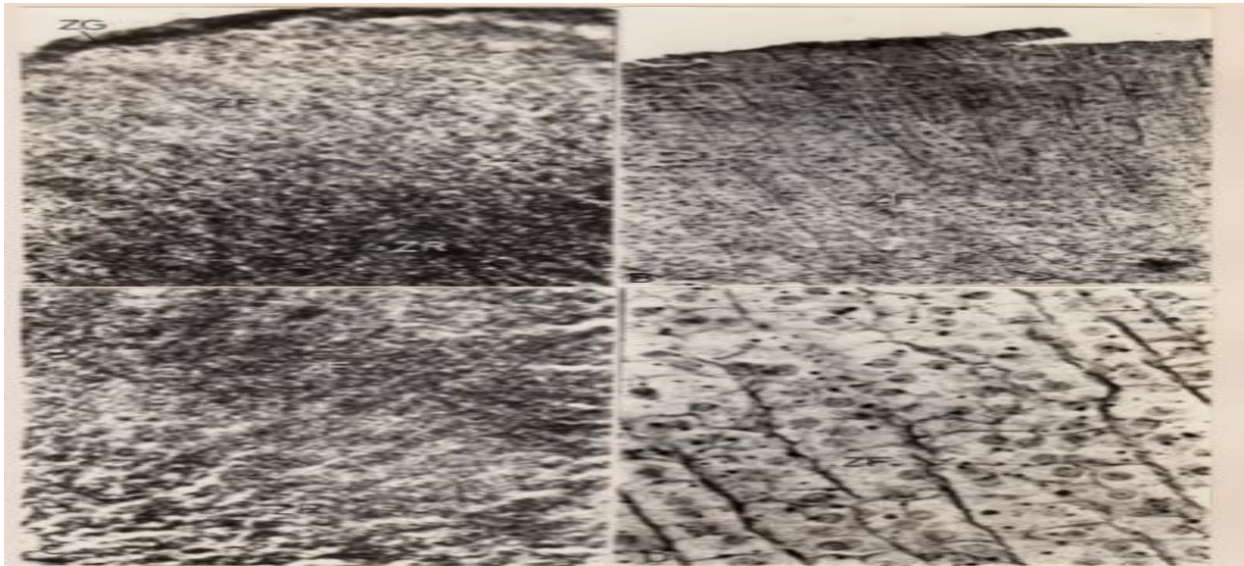


Plate 18 A,B,C&D: Showing localization of AA in the adrenal of rat during early pregnancy.

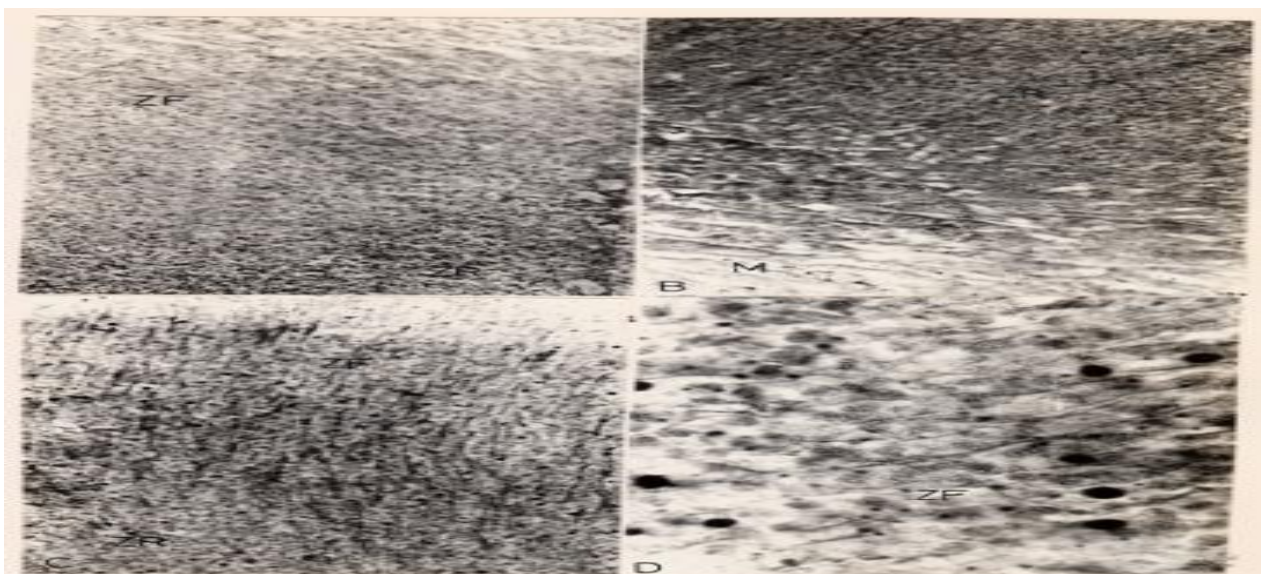


Plate 19A,B,C&D: Showing localization of AA in the adrenal of rabbit during early pregnancy.

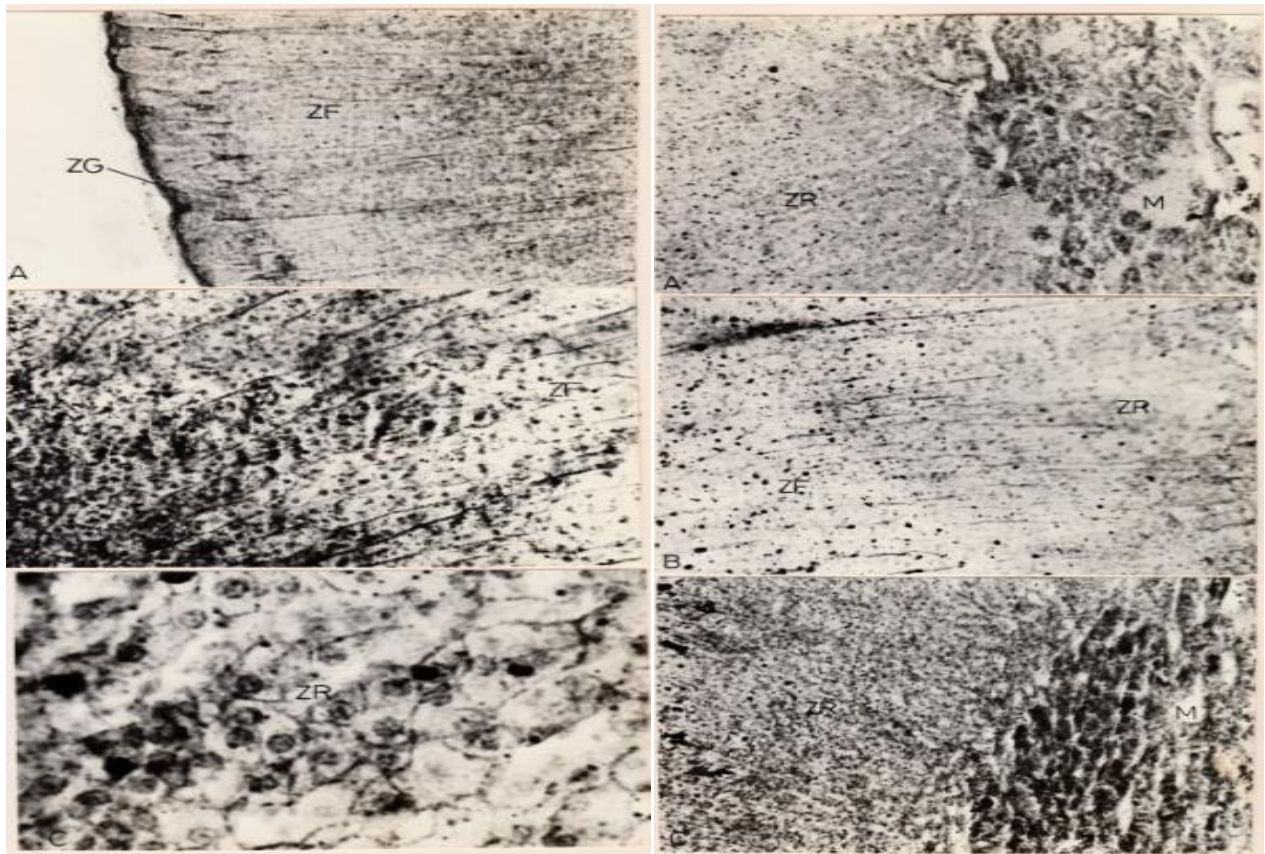


Plate 20 A, B&C: Showing localization of AA in the adrenal of rat at day 6 of pregnancy.
Plate 21 A,B&C: Showing localization of AA in the adrenal of rabbit at day 7 of pregnancy.

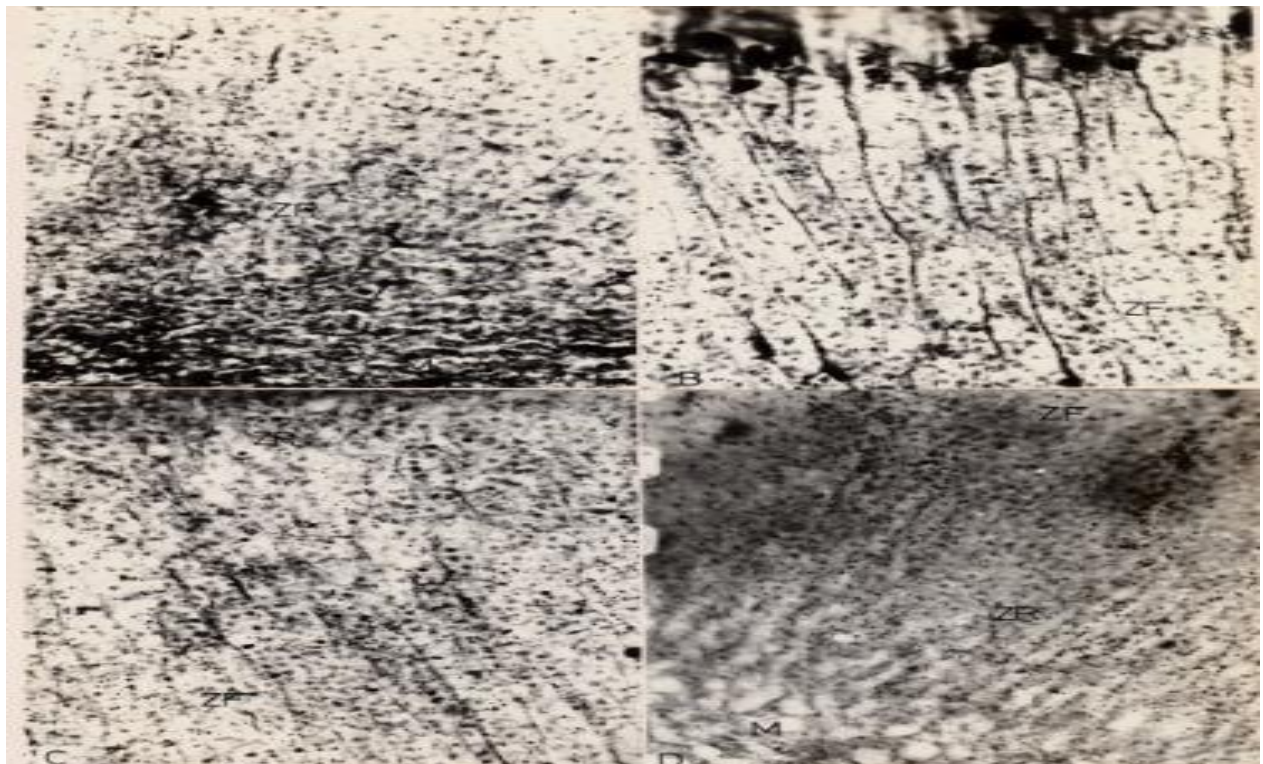


Plate 22: Showing localization of AA in the adrenal of rat & rabbit during later days of pregnancy (at day 12 & 14).

4. DISCUSSION

The adrenal cortical complexes in different classes of vertebrates present enormous morphological Differences. The hormones elaborated by these complexes differ not only quantitatively but also Qualitatively. The adrenal cortex in man, ape and dog produces mainly cortisol, that of rabbit and rat has a massive biosynthesis of corticosterone (Bush, 1955), while that of aves, amphibians and fish elaborates exclusively aldosterone and corticosterone (Carstensen et al., 1961; Sander et al., 1976).

Peroxidase is present in the inner layer of adrenocortical 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 $\Delta^5-3\beta$ – 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).

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.

At the same time, presence of high peroxidase activity and the following depletion of ascorbate during days 5-11 of pregnancy in the adrenal correlates well with stimulated steroid biogenesis in adrenal cortex during this period.

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 to 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

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