CHAPTER 7

HISTOCHEMICAL LOCALIZATION OF ACID AND ALKALINE PHOSPHATASE IN THE GONADS

The function of the phosphatases in the processes such as intermediary metabolism and transport of metabolites across the membrane barriers involving absorptive and secretory mechanisms have been extensively studied. Several workers have studied the phosphatase activity in relation to the fat metabolism as well as the development of gonads in various vertebrate animals. The fact that alkaline phosphatase can catalyze transphosphorylation reaction in addition to its hydrolytic action on organic orthophosphates is well known (Pearse, 1960; Burstone, 1962). Fawcett (1952),reported that alkaline phosphatase appears to be more abundant in the brown adipose tissue than in white adipose tissue. In the brown adipose tissue the endothelium of the capillaries is heavily stained, the nuclei of the fat cells moderately and the cytoplasm faintly. Therefore Fawcett (1952) suggested that the phosphatase in the endothelium is involved in the transport of glucose and fatty acids from the blood stream to the fat cells. George and Eapen (1958) showed a higher level of alkaline phosphatase activity in the adipose tissues of Rosy pastors (Paster roseus) and in the fat body of the desert lucust, Schistocera gregaria.

Fat absorption and acid phosphatase activity in the intestinal epithelium of mice was shown by Barka (1963).

Appreciable concentrations of this enzyme in the cell periphery and in the nuclei of the adipose tissue of the pigeon Columba livia, had been shown by George and Eapen (1959).

It has been shown that phosphatases may be concerned in fructose secretion or in the subsequent metabolism of the sperms, but there appears to be no correlation between fructose content and phosphatase activity, both are, however, influenced by hormones (Gutman and Gutman, 1939; Mann and Parsons, 1947). The presence of phosphatases in sperms has been demonstrated both cytochemically (Friedlaender and Fraser, 1952) and chemically (Mann, 1945) in vertebrate animals. The chemical and histochemical study of alkaline and acid phosphatases in the epidermis of normal, castrated and hormone replaced castrated mice have been carried out by Allan and Slater (1957, 1958). Phosphatase activity in relation to the growth of the testis was studied by Rosenbaum and Lindeman (1958). Electron microscopic studies on the fine structural localization of testicular phosphatases have been carried out by Tice and Barrnett (1963). Again, Niemi and Kormano (1964) demonstrated nucleoside diphosphatase in the spermatids of the rat. The Golgi zone of the spermatogenic cells possessed similar phosphatase activity, to other cells of rat or mouse. The same authors in 1965 reported the cyclical changes in and significance of lipids and acid

phosphatase activity in the seminiferous tubules of rat testis. However, very little is known regarding the phosphatase activity in the crustacean gonads. Alkaline phosphatase and the cycle of nucleic acids in the gonads of some isopod crustaceans had been studied by de Nicola (1949). In the present study alkaline phosphatase ~~as well as~~ and acid phosphatase activities in the gonads of a freshwater crab has been carried out with special emphasis to the cyclic changes in the fat content and to the developmental stages of gonads.

MATERIALS AND METHODS

The freshwater crabs, Paratelphusa jacquemontii, collected from the local rivers were utilized for the study. Testis and ovaries were dissected out and fixed for about 12 to 24 hours in cold 10% formalin neutralized with Sodium hydroxide at 4°C. Tissues were then throughly washed under running tap water and then in distilled water and were embedded in gelatin. 10 µ thick sections were cut on a freezing microtome. After washing in distilled water, the sections were incubated in the incubation medium for the histochemical demonstration of acid and alkaline phosphatase activities, by the Naphthol AS phosphate method described by Burstone (1958a, 1961).

Naphthol AS - BI (Sigma Chemical Company, U.S.A.) was used as the substrate for acid phosphatase activity and

Fast blue B(BBN), (Sigma Chemical Company, U.S.A.) as the coupling salt. The incubation media were freshly prepared and buffered at pH 4.8, 5.2 and 5.4 with 0.2M Sodium acetate. Sections were incubated for 30 minutes to 4 hours at 37°C. In the case of alkaline phosphatase Naphthol AS-MX (Sigma Chemical Company, U.S.A.) was used as the substrate and Fast blue B (BBN), (Sigma Chemical Company, U.S.A.) as the coupling salt. The incubation media buffered at pH 9.2, 8.6 and 8.0 with 0.2M Tris were used. The incubation media and the buffers were freshly prepared. The sections were incubated at 37°C for 16 hours. Out of the several incubation media at different pHs 5.2 for acid phosphatase and 9.2 for alkaline phosphatase gave good results. Two separate controls were used in both the cases. (1) Sections incubated in buffered media devoid of substrate. (2) Sections incubated after keeping in hot water about to boil, for 10 to 15 minutes. After incubation the samples and the control sections were thoroughly washed in distilled water and mounted in glycerine jelly.

OBSERVATIONS

The enzyme localization was indicated by the highly coloured diazonium salt, Fast blue B (BBN). Light blue or violet colour indicated moderate activity and pink or pale

violet showed a very low enzyme concentration. A very high acid phosphatase activity was observed in the fully mature spermatozoa. The sperm nucleus showed a very high enzyme activity, while the cytoplasm was only moderately active. On the whole the spermatozoa appeared to be the site for very high enzyme activity. Just after the completion of spermatogenesis the seminiferous tubules get filled with spermatozoa having very high acid phosphatase activity. Gradually they accumulate in the centre of the tubule (Fig.1). The basal membrane and cytoplasm of the spermatogenic cells also showed remarkably high level of acid phosphatase activity after spermatogenesis, and even when the spermatozoa were still inside the tubules (Fig. 2). The interstitium of the testis was completely negative to the enzyme activity. In the spermatogenic cells the enzyme concentration was more towards the Golgi zone. After the aestivation period the very few spermatozoa which were left out in the seminiferous tubules also showed high acid phosphatase activity, as it was noted just after the spermatogenesis when the tubules were filled with mature sperms. The spermatogenic cells and the interstitium in between the tubules were moderately active during this period. Fat globules which were seen in the interstitial connective tissue, appeared to be moderately stained. A very low enzyme activity was noticed in the spermatogenic cells and in the interstitium in August, i.e. before the starting of the spermatogenesis (Fig. 3), whereas

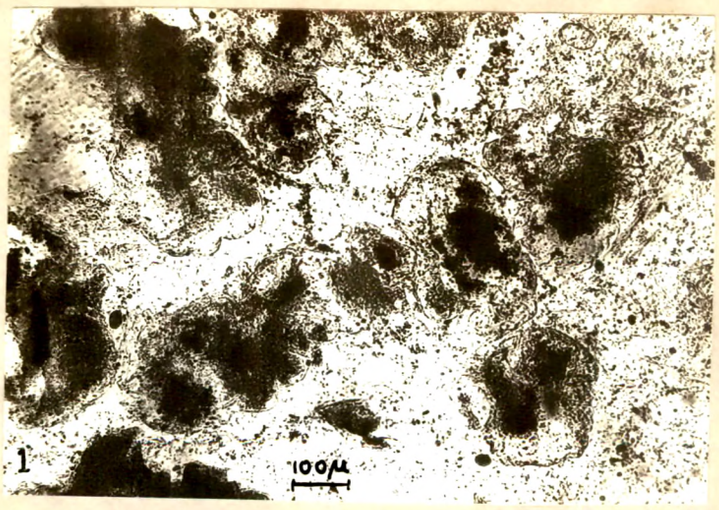
an increased enzyme activity was seen in the germinal epithelial cells and in the spermatozoa after spermatogenesis.

No remarkable acid phosphatase activity was observed in the various stages of the ova formation. Very low enzyme activity was seen in certain fat globules of the fully mature ovum and in the ovarian connective tissue (Fig. 4). In a later stage, before aestivation i.e. in the month of April, small developing ova were seen in between the fully mature ones. During this period, the ovary was completely devoid of any acid phosphatase activity.

The alkaline phosphatase activity of the testis was comparatively less than the acid phosphatase activity. Immediately after the formation of spermatozoa no appreciable activity was observed in them. Only very weak enzyme reaction was observed. Interstitium and the germinal cells of the seminiferous tubules showed uniform, moderate activity(Fig.5). In the spermatogenic cells the activity was localized mainly in the mitochondria. Afterwards when the tubules get filled with the mature spermatozoa, very low enzyme activity was observed in the spermatogenic cells. The interstitial connective tissue was negative to the enzyme activity, but certain fat globules present in the interstitium showed comparatively high activity. In addition to this certain follicles with

dense colloid inside, seen in the interstitium (Chapter 1) also showed moderate activity. In the later stage in March/April, just before aestivation, alkaline phosphatase activity was high in the seminiferous tubules. The basal membrane of the spermatogenic cells showed a moderate reaction for the enzyme activity. The mature spermatozoa in the seminiferous tubules showed a remarkably high activity (Fig. 6). The interstitium was less active. After the aestivation period only a slight activity was seen in the seminiferous tubules.

Low alkaline phosphatase activity was observed in the developing ova. Here the enzyme was extra nuclear in localization (Fig. 7). But the nuclear membrane showed remarkable activity. The ovarian stroma and the egg cytoplasm showed very faint activity in the early stages of development. As the development progressed the peripheral cortical region of the ova showed an increased activity. The connective tissue fibres also showed moderate activity. In the maturing ova fat appeared as large globules at the periphery of the egg cytoplasm. These fat globules were highly active and in the fully mature ova with large fat globules an increased enzyme concentration was noted in the peripheral fat globules (Fig. 8). The alkaline phosphatase activity followed the pattern of fat in these developing ovarian follicles. The vitelline membrane covering the ovarian follicles was devoid.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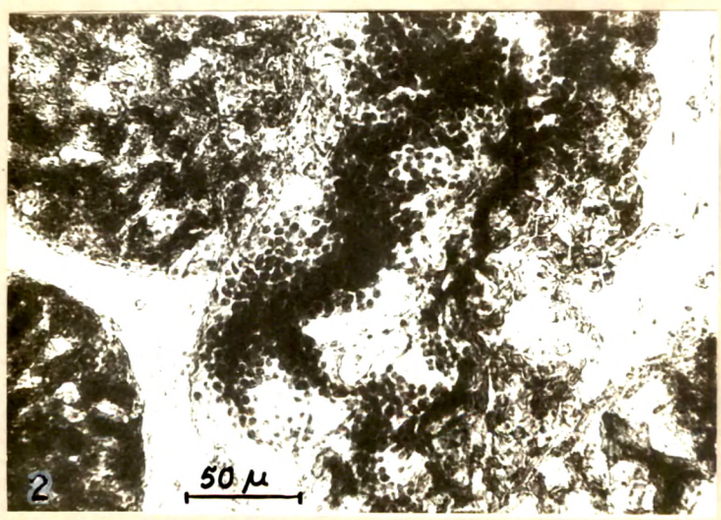
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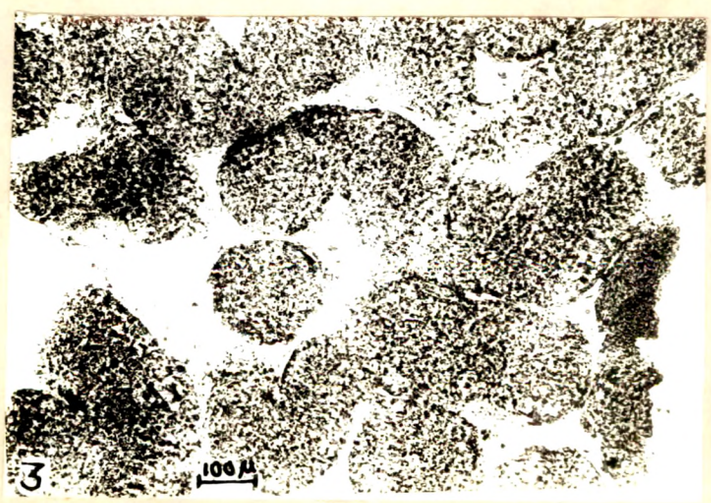
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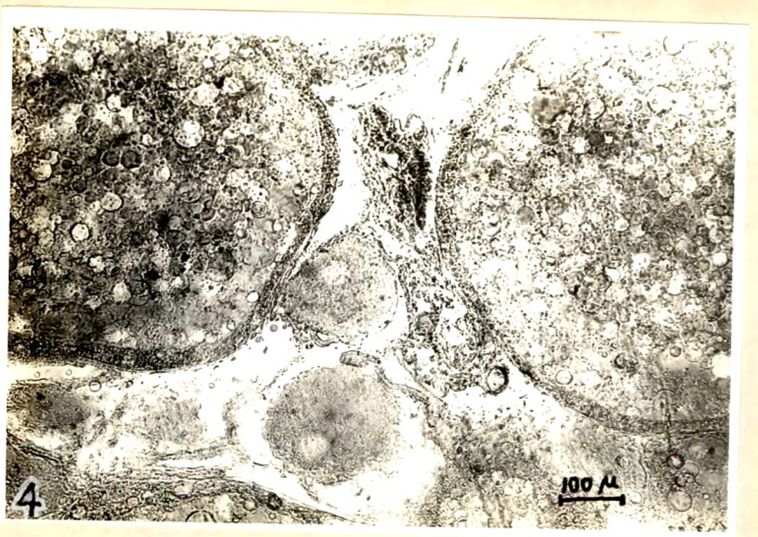
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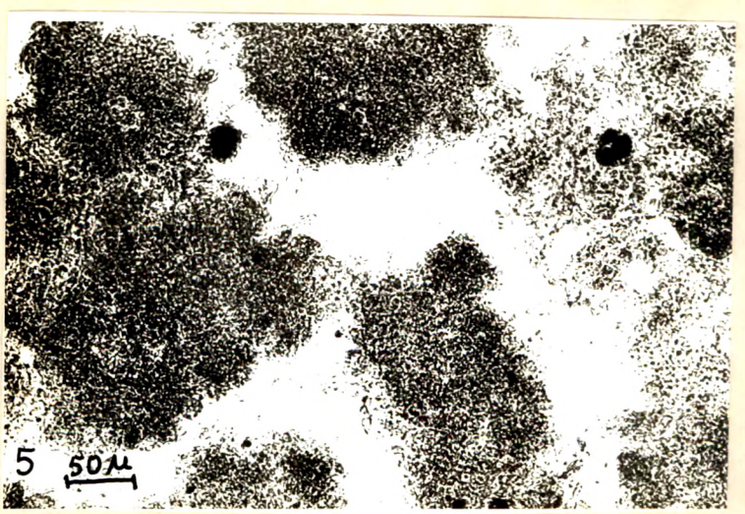
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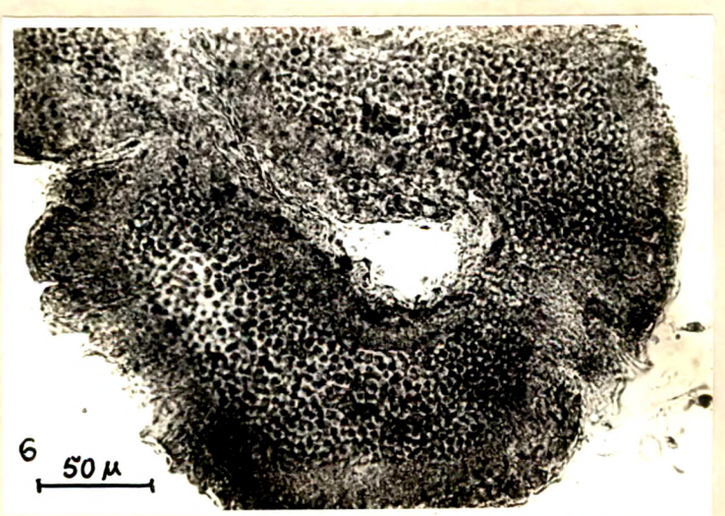
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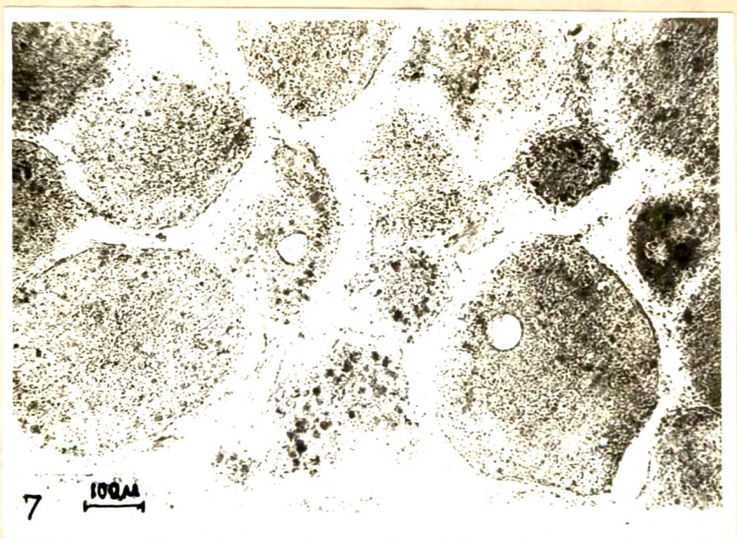
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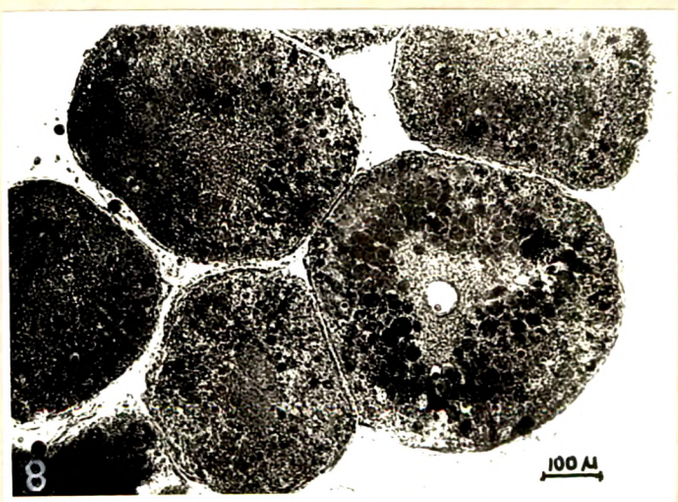
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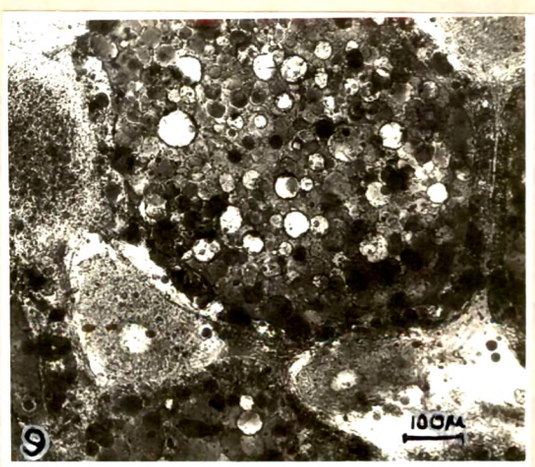
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EXPLANATION TO THE FIGURES

Fig. 1. T.S. of testis showing high acid phosphatase activity in the fully mature spermatozoa in the centre of tubules. Note the uniform moderate enzyme activity in the interstitium.

Fig. 2. T.S. of testis showing high acid phosphatase activity in the spermatozoa. Note the remarkably high enzyme activity in the spermatogenic cells, basal membrane and sperm nucleus.

Fig. 3. T.S. of testis showing very low acid phosphatase activity in the tubules and interstitium before spermatogenesis.

Fig. 4. T.S. of ovary showing very low acid phosphatase activity in certain fat globules of the mature ova and in the ovarian connective tissue.

Fig. 5. T.S. of testis showing uniform moderate alkaline phosphatase activity in the seminiferous tubules. Note the moderately active follicles in the interstitium.

Fig. 6. T.S. of testis showing very high alkaline phosphatase activity in mature spermatozoa in the tubules.

Fig. 7. T.S. of ovary showing low alkaline phosphatase activity in the immature ova and connective tissue. Note the enzyme activity is extranuclear in localization.

Fig. 8. T.S. of ovary showing the high activity of alkaline phosphatase in the developing ova. Note the highly active peripheral fat globules.

Fig. 9. T.S. of ovary showing moderate alkaline phosphatase activity in the germinal epithelium and connective tissue.

of any enzyme activity. The germinal epithelium and the connective tissue showed uniform moderate activity throughout the development of the ova (Fig. 9).

DISCUSSION

It is a generally accepted fact that in a tissue having a high acid phosphatase activity, there is a low activity of alkaline phosphatase or vice versa (Moog,1946). This has also been observed in the case of ovary in the present investigation. The ovary showed a moderate alkaline phosphatase activity while the acid phosphatase activity was comparatively low or absent throughout the development. The testis showed more acid phosphatase than the phosphatase activity in the alkaline range. But in the case of mature spermatozoa both these enzymes appeared to be equally intense. Ambadkar (1965) also observed equal distribution of both these enzymes in rat testis. After the formation of the spermatozoa, a considerably large amount of neutral fat was seen in them (Chapter 5). In these sperms a very high concentration of acid phosphatase activity was noted. At the same time lipase activity was also very high in these sperms (Chapter 6). This suggests the possibility of fat being utilized by the spermatozoa during this period. The presence of neutral fat together with high concentrations of the lipolytic enzyme, lipase, as well as the high acid phosphatase

activity in the sperms shows that both these enzymes are involved in the fat utilization in spermatozoa. Schmidt (1962a), correlated the presence of acid phosphatase in the basement membrane of the epidermis with the metabolism of neutral lipids in the adult newt, Diemictylus viridescens. Further, in the regenrating lizard tail Shah and Chakko (1966) observed a fairly high acid phosphatase activity in those tissues which contained a high concentration of fat. They pointed out that the acid phosphatase is directly or indirectly involved in the fat metabolism in the regenerating tissues. Due to the wide spread distribution of acid phosphatase, Schmidt (1963) has hypothesized participation of this enzyme in the metabolism of several substrates like nucleic acid, protein, carbohydrates and lipids. Shah and Chakko (1966) observed that the sites of acid phosphatase activity were extra nuclear in the regenerating lizard tail. But Burstone (1962), observed that the testis of man, mouse and rat showed an intense activity in the nuclei of spermatogenic elements. In the present investigation also a very high acid phosphatase activity was seen in the sperm nuclei. Even though the spermatophores are being produced in the spermatogenic cells of the seminiferous tubules, they have to travel a long distance to reach the eggs before fertilization. They are passed through the long coiled vas deferens to reach the sperm receptacle of the females, and for this purpose they may require certain amount of energy which they get chiefly

from the oxidation of stored lipids. But ova being lethargic may not need much energy as the sperms. During the development they mainly store fat in the egg cytoplasm. From this it may be inferred that the less utilization of fat in the developing ova may be the cause for the absence or the very low activity of acid phosphatase in the ovary. Only slight acid phosphatase activity was noted in the fully mature yolk ladden egg. Barka (1963) had shown that during fat absorption, in the intestinal epithelium of mice, the acid phosphatase activity was significantly reduced. This indicates that during the process of fat absorption or storage the acid phosphatase activity becomes comparatively very low and helps only in the fat utilization. The low acid phosphatase activity observed in the fully yolk ladden ova again disappears in the later stage, when numerous small developing oocytes start appearing between the mature ones. These young oocytes are under the process of fat absorption, which causes the decrease in the enzyme level.

In the basal membrane and in the cytoplasm of the spermatogenic cells moderate enzyme activity was seen. Activity was more intense towards the Golgi zone of the spermatogenic cells. Tice and Barrnett (1963) reported acid phosphatase activity associated with the Golgi apparatus of the spermatogenic cells and with the dense bodies of Sertoli cells of rat testis, and had suggested that the

enzyme activity may be associated with an exchange of materials between the Sertoli cell and the developing sperm. Biochemical and cytochemical studies have shown phosphatases of the acid variety to have activity against a number of metabolically important phosphate esters (Sumner and Somers, 1953; Bourne, 1954). In the present study the Golgi associated acid phosphatase activity in the spermatogenic cells, soon after the sperm formation and while the spermatophores are still inside the tubule may be involved in the exchange of materials between the cells and sperms. The seminiferous tubules, the interstitium as well as the interstitial fat globules showed uniform, moderate acid phosphatase activity after the aestivation period. During the aestivation period these animals stop feeding and they utilize the stored fat in the body. Immediately after aestivation, when they come out certain amount of fat will be still remaining in the tissue, which they utilize till the next active feeding starts. This continued fat utilization may be the reason for the moderate, uniform activity observed in the seminiferous tubules and interstitium even after the aestivation.

Alkaline phosphatase related to fat metabolism was also studied by various investigators. This enzyme appears to be distinctly more abundant in the brown adipose tissue than in the white adipose tissue (Fawcett, 1952). Wallach and Ko (1964) purified an alkaline phosphatase from aqueous

extracts of rat adipose tissue and they suggested that the alkaline phosphatase of the adipose tissue could conceivably have two functions relative to the synthesis of triglycerides. It regulates the levels of α-Glycerophosphate available for triglyceride synthesis and catalyses a transphosphorylation reaction between free glycerol and suitable phosphate donor to synthesize α-Glycerophosphate. Lipase and alkaline phosphatase were shown to be occurring in appreciable concentrations in the adipose tissue of pigeon (George and Eapen, 1959) and they have correlated the presence of alkaline phosphatase in the adipose tissue with lipid metabolism. A higher alkaline phosphatase activity was observed in the narrow fibres of pigeon breast muscle which contains large quantities of fat (George et al., 1958).

In the present investigation the interstitium and the spermatogenic cells of the seminiferous tubules showed moderate, uniform alkaline phosphatase activity soon after the sperm formation. Considerable amount of neutral fat was also seen in these sites of enzyme activity. The spermatozoa being the sites for high acid phosphatase concentration showed only very low alkaline phosphatase activity. The fat globules present in the interstitium also showed greater enzyme activity, thereby suggesting the role of this enzyme in fat utilization. Certain tyroid like follicles present in the interstitium (Chapter 1) also showed moderate alkaline phosphatase activity. McAlpine (1955) reported a marked increase

in the alkaline phosphatase activity during the differentiative phase of thyroid development. Moog (1962) noted an increased alkaline phosphatase activity in the developing chick embryo in relation to the thyroid and adrenal activity. Just prior to aestivation, the spermatozoa showed a remarkably high enzyme activity. Besides this the whole seminiferous tubules also showed strong enzyme localization. The basal membrane of the spermatogenic cells were also highly active. The fact that alkaline phosphatase activity is closely associated with the transport of the metabolites across the membrane barrier is well accepted. Tice and Barrnett (1963) while studying the localization of the various phosphatases in the rat testis suggested that phosphatase activity may be associated with the exchange of metabolites across the cell membrane. In the present observation a high enzyme activity in the spermatozoa and in the basal membrane of the spermatogenic cells indicates the possibility of alkaline phosphatase being involved in the transport of metabolites across the basal membrane to the spermatozoa.

In the ovary alkaline phosphatase activity followed the pattern of fat. The activity was extranuclear in distribution. Only the nuclear membrane showed certain activity. Several workers have mentioned a positive reaction in the nuclear membrane (Wachstein, 1945; Baud and Fulleringer, 1948; Baud, 1949) in the liver nuclei. Moog and Wenger(1952)

observed that in the ovary of the mouse, the various connective tissue fibres of the theca and the medullary stroma are rich in alkaline phosphatase activity. The same authors suggested that in the ovarian follicle cells the enzyme may well be concerned in the movements of organic substances, into, out of, or across the cells. In the present observations the connective tissue fibres of the stroma as well as the follicles showed moderate activity, which may be correlated to the movements of the organic substances. As the neutral fat globules appeared at the periphery of the egg cytoplasm those seemed to be the sites for high alkaline phosphatase concentration. This is again indicative of the fact that this enzyme is also related to the metabolism of fat in the tissue. However, it should be mentioned that both these phosphatase enzymes are active participants in the metabolism of fat, since in most of the sites they are associated with fat in their localization and distribution, during the various stages of the gonadal development.