CHAPTER FOUR

ACID PHOSPHATASE IN TESTES, OVARIIES, BIDDER'S ORGAN AND OVIDUCTS IN THE ANNUAL BREEDING CYCLE OF THE TOAD, BUFO MELANOSTICTUS.
In comparison with the other vertebrates selected for the present investigation, the toad *Bufo melanostictus* does not exhibit a clear-cut season-specific breeding cycle, but it exhibits continuous breeding throughout the year, though the breeding activities are more intensely seen in the rainy season from June to September. Hence it was rather difficult to find out a definite relation between the acid phosphatase activities and cytologic localization in various reproductive organs and the reproductive events occurring in them, by studying the reproductive organs biochemically and histochemically throughout a year. Hence to get some insight into such relationship, while observations were being taken and recorded, a different approach was made, which is briefly summarised below:

1) Every month a minimum of 15 males and 15 females were studied for the enzyme activities and cytologic localizations of acid phosphatase in various reproductive organs such as testes, ovaries, oviducts and Bidder's Organ in the male.

2) Irrespective of the states of the testes and ovaries, average enzyme activities obtained in all the twelve months of a year were plotted as a function of months, to find out whether the enzyme activities exhibit any annual specific changes.

3) While the monthly observations were being taken on the testes and ovaries, simultaneously small pieces of these organs were subjected to usual histological studies to find out the gametogenic phase of the organs. The enzyme activities and the cytologic
localizations were then considered in relation to such phases. In case of the testes the following staging was done and the observations on the enzyme activities and cytologic localization of the enzyme were then considered with respect to such stages.

1. Testes with seminiferous tubules in which no gametogenic activities were observed.
2. Testes with seminiferous tubules in which active spermatogenesis was in progress.
3. Testes with seminiferous tubules in which degeneration of the spermatozoa in the lumina, and also the degeneration of spermatocytes were observed.

The Bidder's organ was also investigated for acid phosphatase both biochemically and histochemically.

In case of the ovaries also the observations were recorded with reference to -

1. The number of oocytes in which vitellogenesis had not taken place. These are of smaller diameter and did not exhibit any deposition of yolk. These correspond to the previtellogenic oocytes.
2. The number of oocytes in which vitellogenesis had taken place, as could be seen from their larger diameter and cytoplasm full of yolk bodies.
3. The number of eggs full of yolk and with pigment. These are the eggs about to ovulate.
4. Follicles with degenerating eggs. These correspond to the atretic follicles.
In each female toad, the ovary was studied under a magnifying glass and the number of eggs in aforementioned conditions were first recorded. Part of it was subjected to histological studies, a part was utilized for enzyme assay and a part for histochemical techniques.

The oviducts of these females were also separately studied both biochemically and histochemically keeping in view the nature of the ovary as described above. Care was also taken to find out whether the oviducts contain any eggs.

I) Some preliminary Observations:

1. Testes:

Though *Bufo melanostictus* was a potentially continuous breeder, its breeding activities throughout the course of the year were not of the same intensities.

In the case of the males the testicular weights were high in the months of April and May, when their testes were found to be full of mature spermatozoa. With the initiation of rainy season in the month of June, the amplexus seemed to have taken place. At this stage the number of the sperm bundles in the lumina of the seminiferous tubules was found to have decreased, though at no time during this period the lumina were devoid of the sperm bundles. This condition was found to prevail throughout June, July, August and September. October witnessed another surge of spermatogenic activity. Again in the month of February
a third surge of spermatogenic activity seemed to take place which continued through March, April and May.

Thus in toad, though the testes are functional throughout the year and though the spermatogenic activity occurs throughout the year, there are three extensive waves of spermatogenic activity taking place in the months of March, April and May (first wave), October and November (second wave) and February (third wave). Following each surge of spermatogenic activity the sperms seem to be liberated as in the next consecutive month the number of sperm bundles decreases. This is true except for the spermatogenic surge occurring in February.

2. **Bidder's Organ**

On an average more than 80% of the male toads studied in the present investigation showed presence of the Bidder's Organ. One of the significant observations was that this structure was not found in all males studied in the month of July and August, but in the remaining ten months it was regularly noticed and the percentage of the males having bidder's organ was maximum in the month of October and November. Thus at a very general level it appears that when the breeding activities (amplexus) take place at their maximum as in July and August the Bidder's organ is rarely seen, and when such activities decline, though not stop completely, it is seen very conspicuously.

3. **Ovaries**

The oogenesis in the ovaries of these toads was also
found to occur throughout the year, but when the number of pre-
vitellogenic, vitellogenic oocytes, the eggs about to ovulate and
the atretic follicles were examined in the animals collected
every month, it was found that the ovary also passes through
certain waves of oogenic activity. This becomes clear from the
following table:

<table>
<thead>
<tr>
<th>Month</th>
<th>Previtellogenic ova</th>
<th>Vitellogenic ova</th>
<th>Ova about to ovulate</th>
<th>Atretic ova</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>January</td>
<td>70</td>
<td>20</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>February</td>
<td>50</td>
<td>15</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>March</td>
<td>35</td>
<td>25</td>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>April</td>
<td>20</td>
<td>40</td>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>May</td>
<td>25</td>
<td>35</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>June</td>
<td>5</td>
<td>15</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>July</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>August</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>September</td>
<td>5</td>
<td>15</td>
<td>-</td>
<td>80</td>
</tr>
<tr>
<td>October</td>
<td>20</td>
<td>5</td>
<td>-</td>
<td>75</td>
</tr>
<tr>
<td>November</td>
<td>35</td>
<td>25</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>December</td>
<td>40</td>
<td>45</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

From the above table it can be seen that -

1. The oogenesis is very active in the month of May, June, 
   July and August and the ovaries are fully gravid or partly gravid
in the months of June, July and August. In the months of September and October there are no ovulatory follicles.

2. The follicular atresia is significantly seen in the months of September and October. Some atretic follicles can be noted in November and December, but their number was significantly low. In the months of January, February, March and April the number of the atretic follicles was considerably low, whereas in the months of May to August no atretic follicles could be seen in the ovary.

4. Oviduct:

The oviducts on both the sides were conspicuously seen as whitish coiled structures throughout the year. Ova were observed in maximum number in the oviduct in the month of June, July and August. The number of ova within the oviduct decreased conspicuously in September and October. But in November to January they contained ova which were much less in number.

II. Biochemical and Histochemical Observations:

1. Testes:

A) Acid phosphatase activity:

The alterations occurring in the acid phosphatase activity in the testes of P. melanostictus are given in Table No. 2 and plotted as a function of months in a year in Graph No. 2.

The values of acid phosphatase activity expressed as μ moles p-nitrophenol/gm. wet weight of the testes exhibited
interesting alterations throughout a year. In the month of January the acid phosphatase activity was minimum, the average activity being $151 \pm 27$ Units. With the progress of spermatogenesis in February and March the enzyme activity exhibited an increasing trend. The value of acid phosphatase activity was $220 \pm 32$ Units in February and $536 \pm 65$ Units in March. This is then followed by a depletion in enzyme activity to $350 \pm 38$ Units in April and $289 \pm 35$ Units in May. In the month of June, with some early Monsoon showers, the amplexes was initiated and the enzyme activity was enhanced to $450 \pm 53$ Units. The enzyme activity decreased slightly to $371 \pm 42$ Units in the month of July. In the month of August the enzyme activity showed slight increase to $414 \pm 47$ Units and further increase to $505 \pm 62$ Units in September. In the month of October the activity reached a peak value of $1902 \pm 245$ Units. In the month of November the enzyme activity exhibited a sharp depletion to $750 \pm 87$ Units. Further depletion to $312 \pm 35$ Units occurred in the month of December.

B) **Histochemical Observations**

To find out distribution of acid phosphatase in the testis, cytologic localization and changes in the intensities of staining during the testicular activities, the histochemical observations were carried out with reference to - 1) Spermatogenic elements, 2) Leydig cells, 3) Sertoli cells, 4) Spermatozoa.

The histochemically observed cytologic localization of acid phosphatase and alterations in the intensities of staining are shown in Plate No.3, Figs. 1 to 8.
1. **Spermatogenesis**

   The testes in the last week of February and first week of March were found to be in active spermatogenic conditions. Occasionally fully formed spermatozoa were also found in the lumina of the seminiferous tubules. But various stages of spermatogenesis can be made out if several sections are studied.

   During this period acid phosphatase can be made out in the germinal epithelium, though its staining intensity was very poor. Mainly the enzyme was represented by the diffused cytoplasmic staining (Plate No.3, Fig.1). In other stages spermatocytes forming cysts could be observed. In these spermatocytes the acid phosphatase activity was seen mainly in the form of tiny granules occupying a perinuclear zone more towards the plasma membrane (Plate No.3, Fig.3). Such granular enzyme localization in the spermatocytes was a distinctive feature of the testes in the month of March and early April. In late April, various stages of sperm maturation could be observed. In these stages also acid phosphatase cytologic localization was more or less the same as observed in earlier stages. The testes containing mature sperm bundles were observed in June, July, November and in few cases in February. In these sperms the enzyme activity could be detected in the head portion, thus indicating acrosomal localization of acid phosphatase in toad (Plate No.3, Fig.4).

2. **Sertoli Cells**

   Histochemically with naphthol AS-TR technique the acid
phosphatase activity could be distinctly seen in the form of intense granular and cytoplasmic in Sertoli cells during the month of June (Plate No.3, Fig.4). Attachment of sperms in the region of Sertoli cells was also distinctly seen. Intense granular staining was evident in the sperm heads. Similar results are obtained with 6 benzoyl-2 naphthol technique.

3. Leydig Cells:

The Leydig cells exhibited very interesting cytologic localization of acid phosphatase and alterations in the intensity of staining. The Leydig cells could be seen in the interstitium all through the twelve months of the year, but in some months they were very small and comparatively inconspicuous, whereas in others they were quite large and conspicuous. In a similar manner their staining intensity for acid phosphatase also showed similar differences, when they were small and inconspicuous their intensity of staining was very poor, whereas when they were large and conspicuous their intensity of staining was quite high.

The Leydig cells were smaller in size and comparatively inconspicuous in the months of January, September and October. During these months the Leydig cells appeared in the thin sheet of interstitium between the seminiferous tubules. At high magnification acid phosphatase activity in the form of tiny granules could be seen in them (Plate No.3, Fig.1).

In the months of February and early March when the spermatogenic activity was in progress as could be judged from the
presence of cysts of spermatocytes and some stages of sperm maturation, the Leydig cells showed an increase in number, size and also acid phosphatase intensity of staining. The granular staining observed in earlier stages became very clear and evident at this stage (Plate No.3, Fig.2,3). In the months of May, June and July when the testes were found to be full of sperm bundles, the Leydig cells showed maximum development. They increased in number and size. The interstitium was practically full of the enlarged and hypertrophied Leydig cells. Even with 20 min. incubation period in naphthol AS-TR technique they stained so intensely that it was difficult to make out whether the staining was present in the form of diffused cytoplasmic or granular. At high magnification the staining appeared in the form of granules. The number of such granules was so much increased and their staining intensity for acid phosphatase was so high, that they appeared to fuse together forming lumps filling the entire cells (Plate No.3, Fig.6).

In the months of September and October when in the seminiferous tubules signs of lysis were evident, the Leydig cells possessed practically the same localization and intensity of staining as observed in the testes full of spermatozoa. But in this case acid phosphatase positive intense staining, mostly in the form of big granules or small droplets, was visible in the lysing masses of the spermatozoa in the lumina and also in the lysing spermatocytes (Plate No.3, Figs.7,8). Such acid phosphatase staining in the lysing masses of the remnants of spermatozoa mostly resembled the staining of this enzyme during the testicular
regression in the post-breeding periods of other seasonal breeders.

Thus, histochemically the Leydig cells form an important cellular site where distinct alterations in acid phosphatase occur concomitant with the spermatogenesis and the breeding activities of the animals. When the testes are not engaged in spermatogenesis, these cells contain less acid phosphatase, with the progress of the spermatogenic activity the acid phosphatase in these cells increases and reaches maximum when the lumina of the tubules are full of sperm bundles. During some regressive phenomena, which are not very prominent in this animal, acid phosphatase in the Leydig cells does not change much, but with the decline in breeding activities their acid phosphatase content decreases sharply. Mostly this acid phosphatase in the Leydig cells is lysosomal in nature, as could be judged from the typical granular staining.

2. Bidder's Organ

As already mentioned Bidder's Organ was found in more than 80% of the males studied in the present investigation. This structure was not found in the males collected in the months of July and August, when the males exhibit very active breeding activities, but in the remaining ten months of the year this structure was found in about 80% of the males studied. The percentage of males having Bidder's organ was maximum in the months of October and November.

A) Acid phosphatase activity

Acid phosphatase activity in the Bidder's organ showed
certain variations which were statistically very significant. In the month of January the Bidder's organ contained 230 ± 33 Units of acid phosphatase activity, but in February the activity increased to 359 ± 42 Units. March witnessed a significant increase in the enzyme activity, when the activity was 1285 ± 193 Units. In April the activity dropped to 581 ± 77 Units, it varied upto 710 ± 91 Units in May. In June the activity increased to 1282 ± 192 Units, in July and August this organ was found to be absent in the males studied, In September the activity was much lower than June, it was 930 ± 130 Units. In October the enzyme activity further decreased to 565 ± 68 Units. November witnessed a significant increase to 1481 ± 225 Units which was more than six-fold than that observed in January. In December the activity dropped to 454 ± 57 Units.

B) Histochemical Observations:

Bidder's organ was found attached to the anterior end of the testis and histologically it was composed of oocytes in various stages of degeneration or atresia. The number and degree of such atresia differed in different months. At a general level, the number of atretic follicles and advanced stages of atresia were witnessed in the months of March, June and November, whereas they were minimum in the months of January, February and December.

Histochemically acid phosphatase activity was localised in such atretic follicles (Plate No.5, Figs. 8,9). The cytoplasm of the follicles stained so very intensely with 30 minute
incubation period in both the techniques, that it was not possible to distinguish between the granular and diffused cytoplasmic localization of the enzyme. In such histochemically stained preparations, the entire cytoplasm of the follicles was found to be richly crowded with granules of varying sizes, these granules stained positively for acid phosphatase. When such sections were subjected to $\beta$-glucuronidase and esterase localization, it was found that the granules also stained for both the enzymes. The presence of these three lysosomal acid hydrolases in them clearly indicated their lysosomal nature.

Histochemically also the only difference that could be observed in Bidder’s organ concerned with the number of such follicles, the level of atresia and the number and the size of the acid phosphatase-positive granules in the cytoplasm of these follicles. In the months of January, February, April, October and December the Bidder’s organ contained comparatively less number of follicles, contained tiny acid phosphatase positive granules which were comparatively less in number and which also stained less intensely (Plate No.5, Fig.8). On the other hand, in the months of March, May, June, September and November, the number of the follicles was more, they were in advanced stages of atresia and also contained a very high number of these granules, which also stained very intensely. These granules also appeared larger in size and were more or less droplet-like in appearance. They were so heavily crowded in the cytoplasm, that it got stained very darkly (Plate No.5, Fig.9).
3. **Ovary**

The alterations occurring in the acid phosphatase activity in the ovaries of toad are plotted as a function of months in a year in Graph No. 4. In Graph No. 5, the enzyme activities are plotted as a function of the percent previtellogenic, vitellogenic, eggs about to ovulate and atretic follicles, the observations on these made in various months being plotted together. This graph, thus, shows the alterations in the enzyme activities in various functional states of the ovary.

The variations in acid phosphatase activity occurred between 120 ± 25 Units as observed in the month of June and 2208 ± 275 Units as observed in the month of September. In the month of January, when the ovary contained about 70 % previtellogenic, 20 % vitellogenic, 5 % ova about to ovulate and 5 % atretic follicles, the enzyme activity was 145 ± 26 Units. In the month of February this activity increased to 366 ± 42 Units when the percentage of previtellogenic eggs was 50, that of vitellogenic 15, and ova about to ovulate were 30, the percentage of atretic follicles being practically the same. March witnessed further increase in the enzyme activity to 811 ± 98 Units. In this month the percentage of previtellogenic eggs was reduced to 35 and the percentage vitellogenic eggs and the eggs about to ovulate increased to 25 and 35 respectively, whereas the percentage of the atretic follicles remained unchanged. In the month of April when the percentage of the ova about to ovulate remained mostly unaltered but the percentage of previtellogenic eggs
**Table No. 2**

Alterations in acid phosphatase activity in $\mu$ moles p-nitrophenol/gm. in the testes, Bidder's organ, ovaries and oviducts of *Bufo melanostictus* during breeding cycle.

<table>
<thead>
<tr>
<th>Months</th>
<th>Testes</th>
<th>Bidder's organ</th>
<th>Ovaries</th>
<th>Oviducts</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>151 ± 27</td>
<td>230 ± 33</td>
<td>145 ± 26</td>
<td>112 ± 22</td>
</tr>
<tr>
<td>February</td>
<td>220 ± 32</td>
<td>359 ± 42</td>
<td>366 ± 42</td>
<td>208 ± 30</td>
</tr>
<tr>
<td>March</td>
<td>536 ± 65</td>
<td>1285 ± 193</td>
<td>811 ± 98</td>
<td>360 ± 41</td>
</tr>
<tr>
<td>April</td>
<td>350 ± 38</td>
<td>581 ± 77</td>
<td>302 ± 36</td>
<td>228 ± 34</td>
</tr>
<tr>
<td>May</td>
<td>289 ± 35</td>
<td>710 ± 91</td>
<td>209 ± 28</td>
<td>135 ± 25</td>
</tr>
<tr>
<td>June</td>
<td>450 ± 53</td>
<td>1282 ± 192</td>
<td>120 ± 25</td>
<td>331 ± 38</td>
</tr>
<tr>
<td>July</td>
<td>371 ± 42</td>
<td>-</td>
<td>141 ± 27</td>
<td>245 ± 35</td>
</tr>
<tr>
<td>August</td>
<td>414 ± 47</td>
<td>-</td>
<td>672 ± 73</td>
<td>209 ± 30</td>
</tr>
<tr>
<td>September</td>
<td>505 ± 62</td>
<td>930 ± 130</td>
<td>2208 ± 275</td>
<td>116 ± 23</td>
</tr>
<tr>
<td>October</td>
<td>1902 ± 245</td>
<td>565 ± 68</td>
<td>1422 ± 212</td>
<td>790 ± 25</td>
</tr>
<tr>
<td>November</td>
<td>750 ± 87</td>
<td>1481 ± 225</td>
<td>1221 ± 190</td>
<td>298 ± 36</td>
</tr>
<tr>
<td>December</td>
<td>312 ± 35</td>
<td>454 ± 57</td>
<td>190 ± 29</td>
<td>123 ± 25</td>
</tr>
</tbody>
</table>
PV—PREVITELLOGENIC. V—VITELLOGENIC.
OO—OVA ABOUT TO OVULATE.
A—ATRETIC.

ACID PHOSPHATASE ACTIVITY.

2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200

70 PV 35PV 5PV 40PV
20 V 25 V 100 V 45 V
5 00 35 00 80 A 5 00
5 A 5 A 10 A
decreased to 20 and the percentage of the vitellogenic eggs increased to 40, there being practically no atretic follicles, the enzyme activity decreased to 302 ± 36 Units. This decrease was further continued in the month of May, when the enzyme activity was 209 ± 28 Units. In this month 25 % of the eggs were in previtellogenic condition, 35 % of them were in vitellogenic condition and 40 % were about to ovulate, there being no atretic follicles. June witnessed further decrease to 120 ± 25 Units, when the previtellogenic oocytes were just 5 %, vitellogenic 15 % and the eggs about to ovulate were 80 %, there being no atretic follicles. In July and August, the ovary was fully gravid and all the eggs were about to ovulate, in these two months the enzyme activities were respectively 141 ± 27 and 672 ± 73 Units. In the month of September the condition of the ovary changed greatly. There were no ovulatory follicles, 5 % of the oocytes were in previtellogenic and 15 % in vitellogenic condition. The predominant feature of the ovary in this month was presence of about 80 % of the follicles in various stages of atresia. In this month the enzyme activity increased to 2208 ± 275 Units. In the month of October the enzyme activity decreased to 1422 ± 212 Units, when the ovary contained no ovulatory follicles, 20 % previtellogenic and 5 % vitellogenic eggs and 75 % atretic follicles in very advanced stages of atresia. In November the enzyme activity again decreased to 1221 ± 190 Units. In this month only 10 % the follicles were in atretic condition, 35 % oocytes in previtellogenic and 25 % in vitellogenic condition, and 5 % follicles about to ovulate. December witnessed further decrease to 190 ±
29 Units when the percentage of the atretic and ovulatory follicles did not change much, but those of the previtellogenic and vitellogenic oocytes increased to 40 and 45 respectively.

The above alterations in the acid phosphatase activity observed in the ovary throughout the course of a year, thus, seem to be related to the functional states of the ovary. This becomes further clear in Graph No.5. From this Graph it can be seen that -

1) When the ovary contains all the follicles in ovulatory condition, as in the months of June and July, the acid phosphatase activity marks a minimum.

2) When the ovary contains maximum number of atretic follicles, as in the months of September and October, the acid phosphatase activity marks a maximum. It is more than fifteen times the activity seen in the above condition.

3) Leaving aside these extreme cases, in other conditions the enzyme activity varies depending upon the percentage of the follicles in various conditions. At a general level, it can be seen that (a) as the percentage of previtellogenic follicles decreases and the vitellogenic follicles increases, the activity also decreases. (b) As the percentage of the ovulatory follicles increases, the enzyme activity decreases, (c) As the percentage of atretic follicles decreases the enzyme activity also decreases.

B) **Histochemical Observations**

Histochemically acid phosphatase was found to be localized
in the germinal epithelium, cytoplasm of developing oocytes, cortical region of the cytoplasm of fully mature eggs and in atretic follicles. The histochemical picture of the ovary observed in various months of the year varied depending upon the presence of the oocytes and follicles in various conditions.

In the germinal epithelium and tiny oogonia as observed in the months of January, February, November and December, acid phosphatase activity could be visualised in cytoplasm which exhibited very poor diffused staining. Higher incubation periods were essential to visualise this staining (Plate No.4, Fig.1).

In further stages of the development of the oogonia and oocytes, initially cytoplasmic diffused staining made its appearance mostly in perinuclear zone (Plate No.4, Fig.2). At a later stage this cytoplasmic diffused staining gradually turned into granular staining and just prior to the initiation of vitellogenesis the entire cytoplasm was found to be filled by means of tiny acid phosphatase-positive granules (Plate No.4, Fig.3). Yolk nuclei made their appearance in the perinuclear zone during the vitellogenesis and even at this time the entire cytoplasm was filled by means of tiny acid phosphatase-positive granules. When the yolk was gradually deposited, it was observed that in such vitellogenic stages the cytoplasm was filled by a mixture of yolk bodies and the acid phosphatase-positive granules. With the progress of vitellogenesis, these acid phosphatase-positive granules were gradually shifted towards the periphery or the cortical zone (Plate No.4, Fig.4). In the follicles about to
ovulate the perinuclear cytoplasmic region was occupied by the yolk and the peripheral cortical region was occupied the acid phosphatase-positive granules (Plate No.4, Figs. 5,6). These various stages were mainly observed in the ovary in the months of April and May and to some extent even in the month of November.

The corpora lutea-like structure and atretic follicles were observed in the months of September and October. Very intense acid phosphatase reaction in the form of tiny granules and sometimes small droplets was seen in such degenerating follicles (Plate No.4, Figs. 7,8). Even some developmental stages of the follicles also showed atresia and similar acid phosphatase localization. During the follicular atresia, the number of the acid phosphatase-rich particles increased initially in the perinuclear region, these particles, then, were found to have spread all over the cytoplasm of the degenerating eggs. Lysis in the cytoplasm of these eggs was evident. The nuclear material was last to degenerate, where such lysosomal granules were found comparatively late during atresia. These acid phosphatase-positive granules also gave a positive reaction with α-glucuronidase and esterase, which clearly indicated their lysosomal nature.

4. Oviduct:

A) Biochemical Observations:

The alterations occurring in the acid phosphatase activity in the oviducts of toad are given in Table No.2 and these alterations are graphically illustrated in Graph No.6.
Amongst the reproductive organs of toad investigated in the present study the oviducts contained least acid phosphatase activity, which also showed certain alterations during the year. The variations in acid phosphatase activity occurred between 112 ± 22 Units observed in the month of January and 790 ± 95 Units observed in the month of October. The oviducts were conspicuous throughout the year, but the maximum number of ova were observed within the oviducts in the months of June, July and August, whereas their number was found to have decreased considerably in September and October. From November to January, some eggs, much less in number in comparison with June, July and August, were also observed.

In the months of January and February, the oviducal acid phosphatase activities were 112 ± 22 Units and 208 ± 30 Units respectively. March witnessed a significant increase to 360 ± 41 Units. In the month of May the enzyme activity further decreased to 135 ± 25 Units. June witnessed a significant increase to 331 ± 38 Units. In the months of July, August and September the activities decreased to 245 ± 35 Units, 209 ± 30 Units and 116 ± 23 Units respectively. In October the enzyme activity attained a peak level of 790 ± 95 Units. In November the activity decreased to 298 ± 36 Units. In December the activity further decreased to 123 ± 25 Units.

The above described enzymatic alterations, at least at face value, do not show any specific relationship with the functional aspects of the oviduct. But if the ovarian changes are
taken into consideration, it is seen that:

1) When the ovaries contained maximum percentage of the ovulatory follicles and the oviducts contained eggs in them as in the months of June, July and August, the enzyme activities were at higher levels. Same is true for October and November when some eggs were found in the oviducts.

2) When the ovaries contained minimum percentage of the ovulatory follicles and the oviducts contained no eggs, as in the month of January, May and September, the enzyme activities were at lower levels.

Thus, it appeared that the oviducal acid phosphatase activity was mostly related to the passage of eggs through the oviducts. It should be noted here that while the enzyme activities were assayed in the oviducts, care was taken to remove the eggs by taking a longitudinal cut and opening the oviduct laterally. Hence the values obtained for the enzyme activities are due to the enzyme localised in the oviduct itself and not due to the enzyme contained in the eggs in the oviduct.

B) Histochemical Observations:

The enzymorphologic alterations in the oviducts of toad are illustrated in Plate No. 5, Figs. 1 to 7.

During the months of December, January and February the oviducts were comparatively smaller in size and were found as thin coiled structures attached to the dorsal side of the body.
Histologically they showed a thin layer of muscular tissue and a very thin layer of oviducal glands which were smallest in size.

Histochemically with naphthol AS-TR technique moderately diffused cytoplasmic staining was evident in the epithelial cells (Plate No.5, Fig.1). 6 benzoyl-2 naphthol staining also exhibited similar histochemical picture.

During the preparatory period the oviducts showed a distinct increase in their length and diameter. The increase in diameter was found to be due to increase in the lumen and in the size of the oviducal glands. In April the oviducal glands showed considerable enlargement and hypertrophy together with the increase in the length and diameter of the oviducts.

In February acid phosphatase activity increased in the granules located at the basal region of the epithelial cells (Plate No.5, Fig.2). Although there was also cytoplasmic diffused staining, the granules showed an increase in their number. In the month of March the staining intensity increased in oviducal gland cells and mucosal epithelial cells (Plate No.5, Fig.3). Such increase in the staining was much accentuated during April (Plate No.5, Fig.4).

During May and June the oviducal glands showed maximum hypertrophy and the enzyme activity was also maximum. Histochemically with naphthol AS-TR technique both cytoplasmic diffused and granular staining was evident in oviducal gland cells (Plate No.5, Fig.5) and in mucosal epithelial cells (Plate No.5, Fig.6).
Identical staining was also obtained with 6 benzoyl-2naphthol technique.

During October the oviduct was reduced in size, length and weight. The oviducal gland cells and mucosal epithelial cells exhibited intense granular and droplet-like staining (Plate No.5, Fig.7).

These histochemically observed changes agreed very well with the biochemically observed alterations in the enzyme activity.

DISCUSSION

As already mentioned in the Introductory Chapter, excepting a detailed analysis of β-glucuronidase and esterase in the reproductive organs of Bufo melanostictus and Rana tigrina, other acid hydrolases have not been investigated in detail in the reproductive organs of amphibians. From this point of view the present observations find a unique place in the literature on acid phosphatase in the reproductive organs of the amphibians in their physiology of reproduction.

1. **Testis**:

The observations at hand show that though this toad is a continuous breeder and the testes possess a potentially continuous gametogenic activity, there are two distinct and vigorous waves of spermatogenic activity, the first occurring in April and May, and the second in November and December. Accompanying the
spermatogenic activity, changes in the acid phosphatase activity and enzymemorphology are also seen to occur. At a gross level the quantitative alterations in the enzyme activity are well mirrored in histochemically observed alterations in the localization and staining intensity. The low enzyme activity of 151 ± 27 Units in the month of January is mainly due to poor cytoplasmic diffused acid phosphatase activity in the spermatogenic epithelium. The gradual increase in the enzyme activity seen during the preparatory period which attains the level of 536 ± 65 Units in March can be mainly contributed by increase in acid phosphatase activity in the spermatogonia, spermatocytes, spermatids, Sertoli cells and the Leydig cells. During June the biochemically observed activity of 450 ± 53 Units is contributed by the increase in granular staining in spermatocytes, spermatids, sperm heads, Sertoli cells and Leydig cells. In October maximum enzyme activity is observed which is equivalent to 1902 ± 245 Units. At this time intense staining is evident in the lysing masses of remnants of the sperms and Sertoli cells and also the remaining spermatocytes and spermatids which do not differentiate into mature sperms and hence undergo lysis. Intense granular staining is also evident in the Leydig cells. From November onwards sharp decrease in enzyme activity is observed which is reflected in moderate staining in histochemical studies. The changes in acid phosphatase activity can, thus, be correlated with structural changes in the testes and testicular elements containing acid phosphatase activity. Kanase (1979) reported similar variations in testicular β-glucuronidase of B. melanostictus and Mote (1980)
As can be seen from the literature reviewed in Chapter One and also from the final Chapter on comparative considerations, acid phosphatase is not the only acid hydrolysing enzyme found in the spermatogenic elements. Presence of other enzymes such as \( \beta \)-glucuronidase, esterase and aryl sulfatase has been demonstrated in other vertebrates. Acid phosphatase seems to be related to the spermatogenic activity. At what stage of spermatogenesis the enzyme is functioning cannot be explained from the present study. The possible functional significance of acid phosphatase and other lysosomal acid hydrolases during spermatogenesis is discussed in Chapter No. Eight.

In the present investigation the Sertoli cells in the toad testis during the month of June exhibited diffused cytoplasmic and granular staining by naphthol AS-BI technique. During active breeding period the granular staining increased in the Sertoli cells. In the month of October during steatogenesis intense droplet-like staining increased in the lysing Sertoli cells. This was followed by the removal of these cells and new cells differentiated during the preparatory period. Thus, acid phosphatase activity exhibited cyclic variations according to the spermatogenic cycle. Similar cyclic variations in \( \beta \)-glucuronidase in Sertoli cells have been reported in frog (Varute 1969, 1971 a, 1972 a) and toad (Kanase, 1979). Moté (1980) also reported similar variations in esterase in Sertoli cells of frog.

The functional aspect of acid phosphatase and other
hydrolytic enzymes in the Sertoli cells is still obscure. Function of Sertoli cells has been generally described as supplying nourishment to the developing spermatozoa. The morphological appearance of Sertoli cells by electron microscopic studies suggests that these cells might be of importance in providing a link between the extra-tubular vascular supply and the maturing spermatids (Fawcett and Burgos, 1956; Vilar et al., 1962). Lofts (1964), Lacy and Lofts (1962, 1965) and Lacy et al. (1965) have shown that the Sertoli cells of mammals and other vertebrates show a secretory activity, the secretion being that of a steroid hormone, literature on which is reviewed by Mote (1980). Burgos and Vitale-Calpe (1967) studied ultrastructure of Sertoli cells and mechanism of sperm release from Sertoli cells in toad and suggested that the lysosomes present in the cytoplasm of the Sertoli cells may be involved in mucolytic action. The importance of acid phosphatase in Sertoli cells is discussed at comparative level in Chapter Eight.

The Leydig cells form an important cellular site of acid phosphatase localization and the enzymorphology and staining intensity of this enzyme also undergo interesting changes during their various phases of endocrine activity. In the months when the spermatogenic activity in the seminiferous tubules recedes and when the breeding activities also recede, the Leydig cells are not only small and inconspicuous but their acid phosphatase content in the form of tiny granules is also very low. The number of such granules is less and their staining intensity is also poor. When the seminiferous tubules are actively engaged in
spermatogenic activity and when the lumina of the tubules are getting filled by the sperm bundles, and when the toads exhibit heightened breeding activities, the Leydig cells not only increase in number and their size, but their acid phosphatase content also increases significantly. The acid phosphatase-positive granules increase in number and also their staining intensity. When the lumina are full of sperms and when the breeding activities are at their maximum, the Leydig cells are fully enlarged and hypertrophied and their acid phosphatase content in the form of granules also reaches its maximum. In the month of October when the lytic events are taking place in the lumen of seminiferous tubules intense granular acid phosphatase staining was evident in the Leydig cells. When the spermatogenic wave recedes and breeding activities also decrease, the Leydig cells deplete in size and staining intensity.

Similar cyclic changes in \(\alpha\)-glucuronidase have been reported in Leydig cells of *Bufo melanostictus* by Kanase (1979). Mote (1980) also reported similar cyclic variations in esterase content in the Leydig cells of *R. tigrina* during the seasonal breeding-hibernation cycle. Schultz (1973) who studied the ultrastructural alterations in the Leydig cells in *R. esculenta* observed numerous aggregated lysosomes in the breeding period. Varute (1969, 1971a, 1972 c) reported dual localization of \(\alpha\)-glucuronidase in amphibian Leydig cells. The importance of the lysosomal acid hydrolases in the regulation of the secretory function of these endocrine cells is discussed comparatively in Chapter No. Eight.
2. **Bidder's Organ** :

Acid phosphatase is localized in the follicles seen in the Bidder's organ. It is present in the fine granular form, and these granules also stain for β-glucuronidase and esterase, thus, indicating their lysosomal nature. The cytoplasm of the follicles is crowded with such lysosomes and in advanced atretic stages of the follicles such lysosomes increased in number. Presence of such a large population of lysosomes and high acid phosphatase activity in the Bidder's organ is but natural, since these follicles do not develop beyond a certain stage and undergo atresia. Involvement of the lysosomes in such lytic phenomena is proved beyond doubt.

The alterations occurring in the acid phosphatase activity and the lysosomal population in the Bidder's organ seem to be related to the number of follicles and the degree of atresia seen in them. But if the alterations in the enzyme activity are considered in relation to the spermatogenic activity in the adjoining testis, it is seen that, when the spermatogenesis is taking place vigorously in the testes and when the testes are full of sperms, the enzyme activities in the Bidder's organ are at high levels and when the spermatogenesis is not taking place actively then the enzyme activities are at low levels. This further indicates that the follicular atresia in the Bidder's organ is somehow related to the testicular activity or secretion of androgens. In the active breeding when the androgen contents are high, there is marked atresia and higher acid phosphatase
activity. During the period when the breeding activities recede and the androgen levels are low, there is less atresia and less acid phosphatase activity in the Bidder's organ. It seems that acid phosphatase activity in the Bidder's organ is directly depended on the levels of androgen. It will be interesting to study the lysosomal acid hydrolases in the Bidder's organ under experimentally created different hormonal conditions.

3. Ovary:

The ovaries of the toad not only contain an appreciable amount of acid phosphatase activity, but this activity also undergoes certain interesting alterations during the course of a year when the investigations were carried out. The enzyme is localized in the germinal epithelium, oogonia, primary and secondary oocytes and fully mature ova. It is also seen in a very high concentration in the atretic follicles. The acid phosphatase content of the toad ovary depends upon its functional state, and presence of previtellogenic, vitellogenic, eggs about to ovulate and the atretic follicles. When the ovaries contain all the follicles in ovulatory condition, the enzyme activity is at its minimum. When the ovary contains maximum number of atretic follicles the enzyme activity marks a maximum, it is more than fifteen times the activity seen in gravid ovary. Leaving aside these extreme cases, in other conditions the ovarian enzyme activity varies depending upon the percentage of the follicles in previtellogenic, vitellogenic and atretic conditions. As the percentage of previtellogenic follicles decrease and that
of the vitellogenic follicles increases, the enzyme activity decreases. As the percentage of the ovulatory follicles increases the enzyme activity decreases, and as the percentage of the atretic follicles decreases the enzyme activity also decreases.

From the histoenzymological point of view, acid phosphatase shows a dual localization in the granular and the non-granular diffused cytoplasm. The latter localization is seen in the germinal epithelium, oogonia and early stages of developing follicles, whereas the typical granular localization indicating its lysosomal nature is conspicuously seen in the cytoplasmic acid phosphatase-positive granules filling the cytoplasm of the previtellogenic oocytes, which later migrate or are displaced towards the periphery of the oocytes just below the egg membrane during the vitellogenesis when the yolk is deposited. These finally appear in the cortical region of the ooplasm. These resemble the cortical granules described in the oocytes of several other oocytes of different vertebrates. Thus, histochemical observations indicate that the acid phosphatase containing cortical granules are formed in the perinuclear cytoplasm and later on they migrate towards the cortical zone. These granules also gave PAS-positive reaction. Several other enzymes such as hyaluronidase and acid phosphatase (Allison, 1967), β-glucuronidase, acid phosphatase and esterase (Varute, 1969), β-glucuronidase (Varute and Patil, 1970; Kanase, 1979), esterase (Mote, 1980) have been reported in cortical granules of amphibian oocytes.

That these peripheral acid phosphatase-positive granules
are the cortical granules is, thus, established and furthermore presence of a PAS-positive material, acid phosphatase, $\beta$-glucuronidase and esterase in them indicates that these granules are mainly lysosomal in nature. To these biochemical characteristics may be added the presence of hyaluronidase reported by Allison (1967), their membrane bound nature and electron density described by Kemp and Istock (1967), which further substantiate their lysosomal nature.

The cortical granules in the amphibian oocytes have attracted much attention recently and considerable amount of work has been done on their chemical nature and functional aspects, literature of which has been reviewed by Kanase (1979). The present work confirms that though not all, some cortical granules in the oocytes of the toad are lysosomal in nature. The functional role of the cortical granules, a matter of much debate, has to be now essentially visualised in the light of their lysosomal nature. Some ideas along with the opinions expressed by various workers, are discussed in Chapter No. Eight.

In the present investigation atretic follicles were observed. Such preovulatory follicular atresia is more during spawning and postspawning periods. Saidapur and Nadkarni (1973) observed follicular atresia after hypophysectomy in R. cyanophlyctis. They further reported that phagocytic granulosa seems to play an important role in the digestion and disposal of ooplasmic contents of large previtellogenic follicles as well as yolk elements of vitellogenic and mature follicles, whereas
Lofts and Bern (1972) reported phagocytes in atretic follicles in the ovary of *Bufo bufo*.

The typical granular acid phosphatase staining indicating its lysosomal localization in the atretic follicles resembles that observed in the atretic follicles of *T. mossambica* and also of the lizard and bird ovaries described in next two consecutive chapters. The granules are intensely stained and some of them are even of larger dimensions appearing just like the droplets. When such atretic follicles along with intensely staining granules and/or droplets are present in the ovary, the acid phosphatase activity is very high. Thus the biochemical and histochemical observations complement each other. The high acid phosphatase activity and a rich population of the lysosomes in the atretic follicles need no further interpretation. They are directly indicative of the lytic role.

4. Oviducts:

Amongst the reproductive organs investigated in the toad, the oviducts contain minimum acid phosphatase activity. The variations in the activity of this enzyme occurred between 112 ± 22 Units to 790 ± 95 Units. When the alterations in the enzyme activity are viewed in relation to the reproductive stages of the animals, certain curious facts become evident. They are -

1) When the ovaries contained maximum percentage of the ovulatory follicles and the oviducts contained eggs in them, the enzyme activities were at higher levels.
2) When the ovaries contained minimum percentage of the ovulatory follicles and the oviducts contained no eggs, the enzyme activities were at lower levels.

Histochemically the enzyme activity could be detected in oviducal glands and the ciliated mucosa cells. The only histochemically demonstrable alteration that could be noted in the enzyme localization concerns with the changes in staining intensity for this enzyme at these two sites during the passage of eggs. When the eggs are passing through the oviduct, the granular enzyme reaction in the basal half of the cytoplasm of the ciliated mucosal cells increases. On the other hand, when the eggs are not present in the oviduct, the enzyme reaction became moderate, the intensity and the number of acid phosphatase-positive granules decrease considerably.

There are certain comparable studies on \( \beta \)-glucuronidase and other lysosomal enzymes in the oviducts of other amphibians in the literature. In the Indian bull frog \( R. \) tigrina which is a seasonal breeder, Varute (1969, 1971 b) reported that, histochemically and biochemically \( \beta \)-glucuronidase exhibited a distinct increase when the eggs pass through the oviducts, but after spawning the enzyme activity decreases considerably. But in this frog when the breeding period is over the oviduct undergoes regression, during which also the enzyme activity increases considerably, which is localized in phagosome-like droplets and in macrophages. Similar observations for esterase are reported in the oviduct of \( R. \) tigrina by Mote (1980). The toad under
investigation is a continuous breeder and the oviducts remain conspicuous and prominent throughout the year and do not exhibit any regression. Hence the enhancement in acid phosphatase activity observed in such regression is not observed in the toad oviduct.

Because of season-specific alterations in the enzyme activity in the frog oviduct, Varute (1971b) and Mote (1980) could relate such alterations with the alterations in the estrogen and hormones of pituitary origin occurring in the seasonal cycle, but in the toad no such correlation can be established because of the lack of information on hormonal variations.

Tramontana and Polzonelti-Magni (1968) studied acid phosphatase and β-glucuronidase in the oviducts of salamander *Triturus cristatus* during the annual cycle and showed that the activities of these enzymes reach very high levels during the breeding period and also post-breeding period of oviducal regression. The observations that the acid phosphatase activity of the toad oviduct reaches a high level when the eggs pass through the oviduct i.e., the active breeding period, agrees very well with the first part of the observation on the oviduct of the salamander, but the latter part has no parallel in toad oviduct. In the oviduct of toad, *Bufo gargarizana* Chu et al. (1958) observed that acid phosphatase activity increased after the secretion of jelly from the tubular gland and the cells of the tubular gland were immediately repaired and suggested role of acid phosphatase in regeneration of the tubular gland.

De-Martinez et al. (1975) studied oviducal N-acetyl neuraminidase
in the toad, *Bufo arenarum* in which also they reported high enzyme level in the breeding period and low level after spawning. These observations are also in agreement with those made in the present investigation on *Bufo melanostictus* oviduct. Thus, in general, it appears that the lysosomal acid hydrolases in the amphibian oviducts behave in more or less identical manner in their breeding cycles.