Chapter VI - Discussion.
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OVARY PHOSPHOLIPIDS

The various histochemical techniques carried out for phospholipids showed that in the ovary of the fish Labo, the thaba and the interna zona pellucida, the nucleus, the nucleoli, the cytoplasmic granules and the border of the cytoplasmic vacuoles of the egg cells contain phospholipids. The staining of these structures goes on increasing from the youngest stage (I & II) to the fully mature stage (V). These structures and bodies are negative to Sudan black B and haematoxylin after extraction with pyridine and methanol/chloroform, showing that they contain phospholipids.


While studying the ovary of the Labo it was found that only one type of granules lying in the intersices of the vacuoles in the cytoplasm was present. The granules are the mitochondria, or the L1 bodies which contain phospholipids, for they withstand paraffin embedding and dehydration through alcohol after chromation and it is well known that mitochondria contain phospholipids. These granules stained both with Sudan black and haematoxylin (Elftman,
1957 Stain. Technol., 32, 29). They were -ve after extraction with pyridine or methanol-chloroform for 24 hrs. at 60 °C. This establishes the phospholipid nature of these granules. We need not bother about the L3 bodies of Guraya as these bodies consist of triglycerides and will be lost during dehydration as they will not be chromated. The only other bodies that are left are the L1 and L2 which contain phospholipids. The L2 bodies can be left out as these bodies contain a peculiar type of phospholipid and they can only be preserved by fixation in formaldehyde/calcium and postchromed in dichromate/calcium after Baker (1946, Quart. J. Micr. Sci., 87, 441 and Guraya, 1961, Quart. J. Micr. Sci., 102, 381). In this present study none of the above fixatives were used. We are left only with L1 bodies of Guraya or the mitochondria. These L1 bodies, according to Lal (1934, Quart. J. Micr. Sci., 76, 243) who worked on the oogenesis of snakes, are the Golgi bodies. But according to Guraya the nature of these bodies is such that they darken with the Golgi methods and that there is no particular object in these cells to which the name Golgi can be applied.

In the present study one does not come across two types of granules but only one type and these granules are found first diffused in the cytoplasm, but later on they take up a position in the interstices of the vacuoles where they become very clear; there is no increase in the size. They only become more conspicuous as the ovary develops and they are found throughout the cytoplasm. As these granules show all the characteristics of the mitochondria as shown by Guraya in his studies, one may safely conclude that these bodies are the mitochondria.

Chopra (1958, Quart. J. Micr. Sci., 32, 46) in his work on the oocytes of fish, finds two types of bodies, mitochondria and L1 bodies; also according to him the L1 bodies arise from the mitochondria.

This was not encountered in this study.
The nucleoli stain very deeply, as the ovary matures and the number of nucleoli go on increasing. Each of the nucleoli is situated in a sort of a vacuole. The vacuoles show no staining with any of the methods used.

Seshachar & Nayyar (1963, Quart. J. Micr. Sci., 104, 66) pointed out that they did not find any lipids in the nucleoli of the Heteromeuseus fossilis (Teleosti) in their studies of the oocytes and that instead they found intranuclear lipids. In the present study it was found that the nucleoli stain deeply for phospholipids and that there are also intranuclear lipids. From the results obtained in the case of Labo, one cannot agree with the findings of Seshachar and Nayyar that the nucleoli do not contain lipids (phospholipids). In order to establish that it was only the nucleoli that were giving the staining reaction the method of methyl green/pyronine was carried out (Brachet 1942, Arch. Biol., Paris, 53, 207) as modified by Kurnick, 1955, Stain, Technol., 30, 213) and the identity of the bodies that stained was established as the nucleoli. These nucleoli gave a -ve reaction after extraction with pyridine or methanol/chloroform at 60°C establishing the lipid nature of the nucleoli.

It might be pointed out that Serra (1948, Quart. Biol., 13, 192), Albuquerque and Serra (1951, Portugal Act. Biol., 2, 187), Rattenburgh (1952, Stain Technol., 27, 113) Albuquerque (loc. cit.) have reported the successful application of the Smith-Dietrick test and BZL blue to nucleoli after appropriate pretreatment. They also claim that the nucleolar lipids are masked.

(1955, "The Nucleic acids", New York Academic Press, New York) in his studies found small quantities of lipids in the nucleus and that these consist of phospholipids. The finding in this study on Lagen also shows the presence of lipids in the nucleus and these lipids appear to be phospholipids.

According to Subramaniam and Aiyar (1935, J. Roy. Micr. Soc., 55, 174) the nucleolar lipids pass into the cytoplasm. They also reported the formation of nuclear pouches or projections. At no stage in this study were any projections or pouches noticed. It was also noticed that there was an increase in the nucleolar lipids and the lipids in the cytoplasm showing that there is no relationship between the nucleolar lipids and the lipids in the cytoplasm. If there was any relation between the two as suggested by Subramaniam and Aiyar (1935, loc. cit.) the lipids in the cytoplasm should be found round the nuclear membrane; instead they are found diffused in the cytoplasm. But Chopra (1958, loc. cit.) in his work on the oocysts of fish remarks that the $L_1$ bodies and mitochondria are found round the nucleus and that they move towards the periphery, while Seshachar and Nayyar (1963, Quart. J. Micr. Sci., 104, 66) found that they are formed first at the cell membrane and then move towards the nuclear membrane. The findings of Guraya in all his work on lipids in the oocysts show that the $L_1$ bodies are similar to those described by Chopra.

From this one may claim that there might be some relationships between the intranuclear lipids, the nucleoli and the granules in the cytoplasm. But all this has to await further study with labelled isotopes.

The striations of the zona pellucida and the theca interna stain. It might be that the phospholipids are being transported across the zona pellucida from the theca interna to the cytoplasm of the
oocytes. It might then be, as Seshachar and Nayyar (1963, loc. cit.) have found, that the lipid bodies arise near the cell membranes; it may be that these granules are deposited there from the theca interna and, as more are deposited, move towards the nuclear membrane.

It may safely be concluded that this accumulation of phospholipids in the ovary may probably signify the manufacture and discharge of sex hormone. Since phospholipids are found in the theca interna and as it is known that the theca probably produced the hormone, (Hoar, 1957, in "Physiology of Fishes", Academic Press Inc., New York, Pg. 246 and Pg. 287), it might be that phospholipids play a part in hormone production. It may also be that this lipid accumulation might be necessary for some metabolic function. It might be that phospholipids may be required for the developing embryo, as the development is external.

From all this work one can conclude that phospholipids have a definite role in the process of oogenesis.
PHOSPHOLIPIDS (TESTIS)


Molampy & Cavazos (1954, Proc. Soc. Bio. & Exp. Med., 87, 397) worked on a number of animals and they found that phospholipids were present in Leydig cells, sertoli cells, spermatogonia, spermatocytes-primary and secondary and spermatosoa.

In this study on Labeo rohita it was found that phospholipids accumulate in large quantities in the wall of the tubule and spermatogonia cells. As the fish reaches maturity, the primary and secondary spermatocytes and the spermatosoa contain large amounts of phospholipids.

On the basis of this study, it appears that the phospholipids increase from the 1st to the 5th stage. This phospholipid cycle occurs also in the lobule walls, where apparently fibroblasts, in the absence of a true secretory interstitium, probably take on an endocrine function. That the lobule walls are made up of fibroblasts and that they function as an endocrine organ has been pointed by Marshall and Lofts (1956, Nature, 177, 104) in the nile, and Lofts and Marshall (1957, Quart. J. Micr. Sci., 98, 79) in Esox lucius. According to Marshall & Lofts (1956, loc. cit.) in the Labeo the Leydig cells do not arise in the interstitium but in the lobule walls. They also point out that it is impossible to distinguish the immature Leydig cells from connective tissue cells unless appropriate histological methods are used.
The changes in the testis of the *Labae* are very similar to those taking place in the *Esox lucius* (Lofts & Marshall, 1957, *loc. cit.*). But they did not work on phospholipids only, but on total lipids (in general) of the testis. Considering that they used Sudan black the phospholipids should have stained.

In these studies on the *Labae* Elftman's chromation method was used. The material was dehydrated in alcohol and embedded in 56 - 58°C wax. In this process all other lipids are removed in the fat solvents (graded alcohol) and only those that are chromated are left (Pearse, 1960, *Histochemistry, Theoretical and Applied*, Churchill Ltd., London) in the material.

The lipid content of the *Labae* is very similar to that of the *Vipera* (Marshall & Woole, 1951, Quart. J. Micr. Sci., 28, 89). It is also shown in the case of the horned lizards and *Rana mirions* (Melampy & Cavoso 1954, *loc. cit.*) that phospholipids are found also in the cells of the testis. They used Baker's acid haematin method; they did not apply the acid haematin test for phospholipids to the testis of the blue gill, though they tested the testis of all the animals they studied for phospholipids with this method.

The presence of phospholipids in the Leydig cells, as well as in the spermatozoa (middle piece) of the mouse has been reported by Baker (1946, Quart. J. Micr. Sci., 27, 441). These results are comparable to those in the *Labae*. The only difference is the absence of Leydig cells, but the wall of the tubule stains, and as pointed out by Marshall & Lofts (1956, *loc. cit.*) the Leydig cells are found in the tubule wall. In the *Labae* spermatozoa stain but it was not possible to distinguish the portion that stained.
The lipid content is also very similar to that of the mole, *Talpa europaea* (Lofts, 1960, *Quart. J. Micr. Sci.*, 101, 199). In this animal, there is also a gradual increase in the lipid content during the stages of maturation.

From all this work one may conclude that this accumulation of phospholipids in the testis may probably signify the manufacture and discharge of sex hormones. Or since it is a well known fact that phospholipids are necessary for many metabolic functions, this accumulation of the phospholipids might be necessary for some metabolic function. The phospholipids in the spermatozoa might be required to supply the energy for the movement of sperm.

One may therefore safely conclude that the phospholipids have a definite role in the process spermatogenesis.
PHOSPHOLIPIDS (KIDNEY)

The only work on lipids in the kidneys of animals seems to be that of Smith and Freeman (1954, Proc. Soc. Exper. Biol. Med., 86, 775) and that of Modell (1933, Anat. Rec., 57, 13) on the cat. In this study on the kidney in the *Labaeo* phospholipids were studied using the methods of Elftman to demonstrate the phospholipids in paraffin sections. The study revealed that phospholipids are localized in the brush borders, cytoplasmic granules and the nucleoli of the cells of the proximal tubule, in the glomerulus and in the interstitial cells. No lipids were found in any components of the distal tubule cells.

The most notable change in lipids occurs with increasing age, but nowhere was it noticeable that the distribution of the lipids changes from the fine droplets to course droplets as pointed out by Smith (1920, Amer. J. Anat., 27, 69). It was noticed that there was a great increase in phospholipids in the kidney of a fully mature fish the distribution remaining the same as mentioned in the above structures.

It has been pointed out by Wachstein (1955, J. Histochem. Cytochem., 4, 246) that lipids are found in the kidney of the cat and in the kidney of the dog and not in that of any other animal. According to him this will be the task of comparative anatomy to explain these differences in the lipid content of various animals. These differences in the kidney of various species have also been referred to by McManus (1943, Bull. Intern. Assoc. Med. Mus., 24, 73).

According to Wachstein (1955, loc. cit.) the presence of stainable lipids in the kidney obviously expressed disturbed cellular metabolism in man. He also cites the work of Allen (1951 Grune & Stralton, New York) in the kidney of man in various diseases and the
work of Wachstein and Meisel (1953, Proc. Soc. Exper. Biol. & Med., 79, 680) on the effect of toxic substances on the kidney and they find that due to the action of these substances stainable lipids are found in the kidney. This is not the case in the fish studied here, lipids are found in all the stages and that too under normal conditions.

It might be as pointed out by Pillai and Iyengar, 1954, Curr. Sci. V. XIII, 3, 100 that in the lower vertebrates the glomerulus performs both functions of mechanical filtration and secretion, but in the course of evolution. These functions are lost in many vertebrates. It might be that the presence of phospholipids signifies that active metabolism is taking place at all these places where phospholipids are present and that the phospholipids supply the energy required for these processes.

It may be in the case of the brush border that the presence of phospholipids makes it much easier for the reabsorptive function.

The granules found in the cytoplasm of the proximal tubule cells go on increasing till the animal is fully mature. These granules might be the intracellular accumulations observed by Rögaud and Poli-card (1903, C.R. Soc. de Biol., Paris, 55, 216). These bodies definitely contain phospholipids as they are negative to Sudan black B after extraction by pyridine and methanol/chloroform. These granules may be mitochondria for they lie round the nucleus.

The interstitial cells stain for phospholipids. This is confirmed by the fact that they are negative after extraction with pyridine and methanol/chloroform. There is a gradual increase in the phospholipids during the stages of maturation. It might be that
these cells are secretory in function and may be they are under the influence of some hormones. From the presence of phospholipids in these interstitial cells one may conclude that this accumulation of phospholipids signifies the manufacture of some hormones and that these cells are engaged in active metabolism.
Discussion: Krishnamoorthi (1959, J. Zool. Soc., India, 11, 35) has shown, by quantitative chemical estimation of phosphatase, that during the three stages of the gonad of *Labeo* which he chooses for study, the activity rose to peak and that this activity shows a downward trend as complete maturity is attained. In the present study on the *Labeo* it was found that the activity went on increasing and that the peak was reached in the fully mature fish (5th stage).

The enzyme was localized in the nucleoli of all stages, in the cytoplasmic granules and in the border of the vacuoles which are found in the 3rd, 4th and 5th stages. The zona pellucida becomes striated from the 3rd stage onwards. At this stage the zona pellucida attains a great thickness and shows marked radial striations. These striations stain deeply for alkaline phosphatase. The theca interna stains in all the stages.

The staining of the cytoplasmic granules are seen very clearly in the fully mature ovary. The staining of these granules and so also of all the other structures that stain are inhibited by all the inhibitors used, establishing that they are sites that contain alkaline phosphatase.

Kugler (1953, J. Histochem. Cytochem., 1, 296) points out that the enzyme is present only in the follicular epithelium and not in the ovaries proper. He also further states that a positive reaction is observed even in control sections with the same amount of
intensity as in the experimental sections. Such a staining was not encountered in this work. In the Labeo control sections which were incubated without substrate, and after boiling showed no staining at any of the sites that previously stained.

The work of Osawa (1951, Embryologia, 2, 1) on amphibian eggs was in keeping with the work of Kugler (1953, loc. cit.). Osawa (1951, loc. cit.) in his work only used the Takamatsu and Gomori methods. In the present study the methods used by Osawa and also the naphthal methods of Burstone were used and they gave good results with all the stages.

Venugopalan (1961, Expt. Cell Res., 24, 1) in his study on the Notonteris found that the enzyme activity is absent in the cytoplasm in the early oocytes, similar to the observations of Krugelis (1947, Biol. Bull., 93, 309 and 215) in other animals. In the present work on the Labeo the cytoplasm of the immature stages shows a diffused staining with all the methods used and this staining is inhibited by the various inhibitors.

Krugelis (1947, loc. cit.) in her work on marine invertebrates, Arabacia, Asterae etc. found phosphatase activity in the germinal vesicle of the oocytes including the nuclear sap and nucleoli, but no alkaline phosphatase was detectable in the cytoplasm. She also made similar observations on the growing oocytes of Rana, Axolotl and Ambystoma (1947, loc. cit.). Ramamoorthy (1959, Proc. Natl. Inst. Sci., India, 25, 87) has reported that in the Pila virans the sites of phosphatase activity in the cytoplasm are in the form of granules in the peripheral region in the fully grown egg. In the case of Labeo the granules were found in the interspaces of the cytoplasmic vacuoles. The border of the cytoplasmic vacuoles also stained showing the presence of alkaline
phosphatase in the border of the vacuoles; the inner portion of the vacuole remains unstained. The nucleoli also stain and the staining goes on increasing till the maximum is reached at maturity. Lison (1948, Bull. histol. appl. physiol. et path. et tech. microscope, 25, 23) and Ruyter and Newman (1949 Biochem. Biophys. Acta, 3, 125) point out that non-enzymatic staining of yolk, nucleoli and chromosomes is caused by the adsorption of cobalt. This might be the case when the Gomori method is used but when the naphthol methods are used as in this study no such claim could be made. That alkaline phosphatase is a nucleolar constituent is a widely accepted fact (Danielli, 1953, Cytochemistry, Wiley, New York and Bradfield, 1951, Quart. J. Micr. Sci., 22, 87) and generally the nucleolus is one of the most intensely stained area in a phosphatase preparation. Tandler (1953, J. Histochem. Cytochem., 1, 151) found that the nucleoli of several tissues have an affinity for the bivalent ions used in the phosphatase reaction and thus will give a positive reaction irrespective of the amount of enzyme present. Vincent (1952, Proc. Nat. Acad. Sci., U.S., 32, 139) found that the nucleoli of the starfish oocyte gave intense staining for alkaline phosphatase in tissue sections, but no activity could be detected for this enzyme on direct analysis of isolated nucleoli.

Brachet (1944, Enzymologia, 11, 336) by quantitative methods found small amounts of alkaline phosphatase in isolated nuclei of amphibian eggs which could not be detected by histochemical methods; this in no way denies the presence of alkaline phosphatase.

Venugopal (1961, Expt. Cell Res., 24, 3) found that the nucleoli contain a large amount of alkaline phosphatase and that the nucleoli go on increasing in number with maturity, but on maturity there is a decline in the alkaline phosphatase. In the present
investigation on *Laboa* alkaline phosphatase was found to increase to a maximum on maturation, but it was not possible to establish whether the nucleoli increased in number.

The fact that the nucleoli contain alkaline phosphatase shows that the nucleoli like the granules in the cytoplasm are engaged in active synthesis concerned with the liberation of finished proteins in sites of nucleic acid and especially in the nucleoli (Bradfield, 1951, *Quart. Micr. Sci.*, 22, 87). It can also be that these sites of high alkaline phosphatase are sites of active transport. The nucleoli may be responsible to supply the developing embryo with all the required nourishment.

Venugopal (1961, *loc. cit.*) has nowhere mentioned that the zona pellucida shows striations and that these striations show alkaline phosphatase. The same is the case with the work of Kugler (1953, *J. Histochem. Cytochem.*, 1, 296). The study of the *Laboa* oocytes showed that the zona pellucida has striations and that these show intense alkaline phosphatase activity which went on increasing from the 3rd stage (a stage at which the striations become visible for the first time) to the 5th stage (which is the fully mature stage).

It might be that these striations must be sites of active transport, since they contain alkaline phosphatase, and that the various substances that are produced outside the oocyte are transferred to the developing oocyte and that as the development proceeds a large quantity of materials are transported across the membranes.

Theca interna stains deeply and there is a great increase of alkaline phosphatase till the ovary is fully mature, the highest
intensity being in the fully mature ovary. This localization of alkaline phosphatase in the theca interna is in agreement with the work of other workers. Thus Deane (1952, Amer. J. Anat., 91, 363) found that alkaline phosphatase was present only in the capillary wall of the theca interna of normal follicles.

Here in the Labeo it was found in the whole theca interna. This high content of alkaline phosphatase is characteristic of sites of transport mechanisms. The theca interna lies between the egg and the blood vessels that supply different constituents for the various synthesis of proteins, lipids and carbohydrates by the oocytes. It may be that this intense activity of the theca is concerned with the transport of solutes to the growing oocyte. The alkaline phosphatase in these various sites in the ovary may supply the required energy for the breakdown of various materials needed by the developing embryo; that may be the reason why the maximum intensity is found in the fully mature ovary.


In this study of the Labeo the esterases were found localized in the granules in the cytoplasm in the oocytes, the border of the vacuoles in the cytoplasm and in the zona pellucida. In the case of the zona pellucida, the striations do not stain alone but the whole zona does.

From the action of eserin sulphate on the esterase of the ovary it may be safely concluded that cholinesterase is present in
very small amounts. The esterases are of the non-specific type since they are inhibited by sodium fluoride. When the material was tested for lipase it was found that lipase is altogether absent in the ovary. This is in agreement with the finding of Barrnett's data (1952, loc. cit.) that all the esterases of the ovary are of the non-specific type and that true lipase is absent.

Hunter and Kneiske (1957, J. Histochem. Cytochem., 5, 154) found no esterases in the rat ovary after fixation with acetone. It appears that acetone destroys the esterases which are readily demonstrated with short chain esters of naphthol and naphthol AS. This was also the case with Malaty and Bourne (1954, Acta Anat., 21, 246) in their study of the esterases in the human ovary. In the present study of the Labes acetone was used and the staining was the same as when other fixatives were used. Markert and Hunter (1959, J. Histochem. Cytochem., 7, 42) separated out the esterase of the mouse ovary and got a number of bands on their zomograms. It has also been pointed out by them that increasing maturity is accompanied by increasing types and amounts of esterases. In the case of Labes too it was found that the amount of esterases progressively increased in the maturing ovary. It was, however, not possible to point out if there was an increase in the types of esterases.
From the study carried out on alkaline phosphatase, esterases and lipases in the testis of the Lahao, one may arrive at certain conclusions.

Alkaline phosphatase was localized in the wall of the tubule and it increases from the immature to the fully mature or pre-spawning assemblage. This increase was visualized by Gomori's ca-co & azo dye methods and by Burstone's method using Naphthol AS--MX & LC phosphates. From the results it may be concluded that the enzyme present shows no substrate specificity indicating that the alkaline phosphatase is of the non-specific type.

The presence of alkaline phosphatase in the tubule wall signifies that metabolism is taking place at these sites. It may be that alkaline phosphatase is engaged in synthesis of other chemical substances. This high contents of alkaline phosphatase may be on account of the active transport taking place in the wall of the tubule. It has been pointed out by Moog (1946, Biol. Rev., 21, 41) and Lipman (1941, Advances in Enzymology, 1, 99) that alkaline phosphatase is a characteristic of sites of transport mechanisms.

It may be that since the wall of the tubule lies between the spermatic elements and the blood capillaries that supply different constituents for the various synthesis by the spermatic elements, this wall of the tubule is concerned with the transport of solutes to the spermatic elements.

This alkaline phosphatase cycle which occurs in the lobule wall may be due to the fibroblasts which in the absence of true
secretory interstitium probably take on an endocrine function. That
the lobule wall is made up of fibroblasts and that its function as
an endocrine organ has been pointed out by Marshall & Lofts (1956, 
98, 79).

This increase in alkaline phosphatase may signify the dis-
charge of a sex hormone. That the increase is greatest in the fully
mature testis, shows that this accumulation of alkaline phosphatase
is under the influence of some hormones. The influence of hormones
upon the enzymatic activity of the connective tissue has also been
noted by Bern (1949, Anat. Rec., 104, 361). It may be that after the
discharge of the spermatogenic elements, this alkaline phosphatase causes
the discharge of hormones signalling the start of a new spermatoge-
genous cycle.

Esterases: The presence of esterases in the testis of Lbarn was
studied by the use of α-naphthyl acetate, naphthol AS - NX and
naphthol AS - LG acetate. The results showed that esterases were
localized only in the wall of the tubule, no matter which of the
substrate was used. These findings that esterases react similarly
to the substrates is in keeping with the work of Pearce (1954,
Inst., 19, 167) on the tissues of rat.

The reaction of inhibitors on the esterases is as follows:
when eserine sulphate (10⁻⁴ M) was used, there was no inhibition;
this was expected for eserine is a cholinesterase inhibitor and
cholinesterase is supposed to be absent in the testis. The other
inhibitor used was sodium fluoride (10⁻⁴ M); with this inhibitor
the inhibition was complete, further showing that the esterases were
of the non-specific type.
Many workers have found esterases in the testicular Leydig cells (Nachlas & Seligman, 1949, Anat Rec., 105, 677; Chessick, 1953, J. Histochem. Cytochem., 1, 471 and Hayashi et al., 1959, Acta Path. Jap., 2, 113). But in the case of *Laboa* the Leydig cells do not arise in the interstitials but in the lobule wall (Marshall & Loffs, 1956, loc. cit.). It is might be on account of this that the staining in the *Laboa* is localised in the wall of the tubule.

According to Market & Hunter (1959, J. Histochem. Cytochem., 2, 46) there are only three zones of esterase activity in their electrophoretic studies on the mouse testis. While Niemi et al. (1962, J. Histochem. Cytochem., 10, 2) in their electrophoretic studies found eight zones of activity. From this it can be established that the non-specific esterases are made up of a number of esterases. Niemi et al. (1962, loc. cit.) concluded that the testis contain both A & B type esterases. It was not possible to study the esterases of *Laboa* by electrophoresis and to use various inhibitors on account of lack of availability of inhibitors.

It is a little difficult to assign any definite role to esterases. The biochemical properties of this enzyme indicate their activity in some phase of lipid metabolism (Ammon and Jaarsma, 1953, in "The Enzymes", Ed. Sumner & Myhrk, Academic Press, New York, p.390).

Niemi et al. (1962, J. Histochem. Cytochem., 10, 186) claimed that there is no true lipase in the testis of rat, but only non-specific esterase because of their negative results with tween 80.
In this study of the testis of the fish, *Leboc*, it was found that a true lipase is present. The method used was that of George and Iype (1960, Stain Technol., 35, 161). The fixation of the material was done in acetone and chloral hydrate Formalin. The sections were pre-incubated in sodium taurocholate 0.1M for ½ hr. and 1 hr. and then the Gomori (1952 "Microscopic Histochemistry" Univ. Press, Chicago) and Martin's (1955, Nature 172, 1043) method for staining were followed. Only those sections that were preincubated for 1 hr. stained with Tween 20 and Tween 80. It is known that sodium taurocholate is an inhibitor of non-specific esterases and that this staining must be due to lipases. It is also known that a "true lipase" should be able to split esterases of unsaturated fatty acids; the non-specific esterases have the same capacity but work at a slower rate (Gomori, 1949, Proc. Soc. Exp. Biol. Med., 72, 697). Of the tween series used in this study only Tween 80 contains unsaturated fatty acid (oleic acid) and it is just the one that stained showing the presence of true lipase.

Long and Engle (1952, Ann. N.Y. Acad. Sci., 55, 613) and Nagashi et al. (1959, Acta path. Jap., 8, 113) reported the presence of lipase activity in the Leydig cells of rat and man. In the present study if lipase was to be found in the Leydig cells it should have been found in the tubule wall. But this is not the case; here lipase is found in the cellular elements of the seminiferous tubules. It is also remarkable that in all the cellular elements of the seminiferous tubules no staining for lipase was obtained with the original Gomori method, but only on preincubation with sodium taurocholate and then using the Gomori method a staining was obtained. It may be that the lipases present require an activator.
According to George and Scaria (1968, J. Animal Mor. & Physiol., 5, 43) acetone is known to inhibit lipase activity completely. This was not the case in this study in which acetone was used. On the ground of acetone being an inhibitor George and Ambedkar (1963, J. Histochem. Cytochem., 11, 420) criticize the work of Niemi et al. (1962, loc. cit.) who used acetone as a fixative. But from this study of Lasan it seems to show the contrary, that acetone is as good a fixative for lipases.

Niemi et al. (1962, loc. cit.) should have used some activators in the study of lipases before making such claims as to the absence of lipase.

The presence of this enzyme in the cellular elements of the seminiferous tubules indicates that active lipid metabolism is taking place in these elements.
KIDNEY ALKALINE PHOSPHATASE

Histochemically alkaline phosphatase has been demonstrated in the kidney of most vertebrates with the exception of Amblystoma, maculatum, Chelydra serpentina and Crocodylus tanneri etc. mentioned by Wilmer (1944, Arch. Pathol., 37, 227). He was unable to demonstrate the presence of alkaline phosphatases in the kidney of these animals.

Wilmer (1944, loc. cit.) interpreted these findings as a valid evidence of the relation of the tubular enzyme to glucose transport. It is generally accepted that glucose undergoes phosphorylation in the process of absorption from the glomerular filtrate (Shannon, 1942, Ann. Rev. Physiol., 4, 297). The formation of a hexose phosphate ester is presumed to create the diffusion gradient which permits a continuous flow across the tubule membrane (Kalcher, 1941, Chem. Rev., 22, 71). This ester is hydrolysed and the glucose discharged into the peritubular blood stream.

Kalcher (1937, Skand. Arch. F. Physiol., 77, 46) found large quantities of adenosine triphosphatase and alkaline phosphatase within the cells of the proximal tubules. Possibly phosphorylation is effected at the expense of the labile phosphate bond energy of ATP since these two enzymes are necessary for phosphorylation of glucose and hydrolysis of the ester. It is known that glucose is absorbed by cells of the proximal segment (Walker, 1941, Amer. J. Physiol., 134, 580 and Walker and Hudson, 1937, Amer. J. Physiol., 118, 130) which contain alkaline phosphatase in the characteristic localization in the brush border (Gomori, 1941, J. Cell & Comp. Physiol., 17, 71).
In this study on the \textit{Labeo} alkaline phosphatase was found in the brush border and nucleoli of the proximal tubule, interstitial cells and the glomerulus. If alkaline phosphatase is to be concerned only with the hydrolysis of the hexose ester formed within the tubular cells in the reactions mentioned above, it should be absent in those forms which possess no glomeruli, filter no glucose and have no system for the reabsorption of glucose. Wilmer (1944, \textit{loc. cit.}) pointed out that alkaline phosphatase is absent in the renal tubule of the toad fish, and agglomerular marine teleost. While as is seen in a fresh water glomerular teleost it is in abundance.

Kalchar (1941 \textit{loc. cit.}) has pointed out that the agglomerular toad fish has less phosphatase than the glomerular kidneys from closely related species. If the observations above are correct, then there is strong evidence that alkaline phosphatase in the fish is largely concerned with the reabsorption of glucose, for the urine of glomerular and agglomerular fishes does not differ appreciably in composition. (Marsh, 1930, Amer. J. Physiol., 1, 94).

Danielli (1953, \textit{Cytochemistry,} Wiley, New York and 1954, Proc. Roy. Soc., B. (Lond.), 2, 142) has some doubts about this theory and has made mention of the unpublished studies of Lorch and himself revealing the presence of tubular alkaline phosphatase in a number of species of agglomerular fish. Also from the studies of Longley (1956, Science, 122, 595), it can be said that all this argues against the involvement in glucose resorption and to some other tubular function.

A review of the literature of alkaline phosphatase in the kidney of the animals studied by Wilmer (1944, \textit{loc. cit.}) seems not
to mention any staining of the glomerulus. Under pathological conditions it is supposed to be found in the glomerulus. Wachstein (1944, Arch. Path., 33, 297) and Soulairac (1948, Comp. Rend. Soc. Biol., 142, 297) working on diabetic rats found that alkaline phosphatase was absent in the glomerulus while Kar & Ghosh (1951, Sci. & Culture, 17, 175), found it in abundance in the glomerulus of diabetic rats.

Pillai & Iyengar (1954, Curr. Sci., 23, 100) reported the presence of alkaline phosphatase in the glomerulus of Rana banadensis. The same was the case with Labeo and it was also found that though the glomerulus contained less alkaline phosphatase than the brush border yet there was a corresponding increase of alkaline phosphatase in the glomerulus along that in the proximal tubule. It is in agreement with the work of Pillai & Iyengar (1954 loc. cit.) that it might have been that the glomerulus played a dual role of mechanical filtration and secretion and that it has lost it in its evolution in many vertebrates, though still maintaining the potentialities for enzymatically controlling the output of filtrate.

Since alkaline phosphatase is vital not only for the formation of bone, but also for the metabolism of carbohydrate, nucleotides and phospholipids, (Summer and Somers 1953 Chemistry and methods of enzymes). It might be that these structures that contain alkaline phosphatase are involved in active metabolism and that the enzymes may be part of a whole enzyme system evolved in the liberation of the newly synthesised protein from a complex with nucleic acids (Bradfield, 1951, Quart. J. Micro. Sci., 82, 87). It may also be that active transport of materials is taking place across these structures.
Kidney Esterase: The esterases were found in the cytoplasm of the cells of the proximal tubule. It was not possible to establish if the enzyme was localized in the cytoplasm as granules, as the whole of the cytoplasm stained. The pattern of staining was the same with Naphthol AS - LC acetate, Naphthol AS-MX acetate but with Naphthol AS-acetate, there was no staining, the action of eserine sulphate on the esterases was very little, showing the presence of only small traces of cholinesterase. The action of sodium fluoride was very marked, the tests were negative, indicating the presence of non-specific esterase. These findings are in keeping with the work of Wachstein (1955, J. Histochem. Cytochem., 3, 243).

The absence of esterases in the glomerulus is in keeping with the work of Wachstein (1955, loc. cit.) and Chessick (1953, J. Histochem. Cytochem., 1, 471). Glenner and Marvin (1958, Anat. Rec., 130, 2) found that esterases were present in the glomerulus of Nacturna maculosa; they also found certain differences in the sites of activity, when two different substrates were employed. Their work is in keeping with the work of Gomori (1952, "Microscopic Histochemistry, Principles and Practice," Univ. Chicago Press, Chicago, p.154) and Chessick (1953, loc. cit.).

The findings of Glenner and Marvin (1958, loc. cit.) were never encountered in the kidney of Laha. All substrates revealed the same sites of activity. With all the substrates and activator, (cystine), no other sites of activity were noticed. Wachstein and Neisal (1957, J. Histochem. Cytochem., 5, 204) reported that esterases were found in the proximal and distal convoluted tubule, in the ascending limb of Henle's loop and in the collecting tubules, when unfixed frozen sections were used. In this study only the proximal tubule
stains; the other components mentioned by Wachstein and Meisel (1957, loc. cit.) do not.

It was not possible to classify the esterases, by the use of various inhibitors as mentioned by Skinkta and Seligman (1961, J. Histochem. Cytochem., 2, 504) on account of the non-availability of inhibitors. Lipase was not present in any of the kidney components. The reaction was -ve after treatment with sodium tauro-
cholate, which according to George and Ambedkar (1963, J. Histochem. Cytochem., 11, 420) gives better results for lipase.

It is difficult to assign any particular physiological function to the esterases in the kidney. It may be that the esterases play some part in lipid metabolism, for it has been shown that esterases decrease along with the disturbed lipid metabolism which occurs in renal diseases - (Svanborg A 1953, act. med. Scand., 142, 349 and Wachstein, 1946, J. Exptl. Med., 84, 25).
Chapter XI - Discussions.
OVARY: Histochemical reactions of the developing ovary reveal the presence of large quantities of PAS+ve material in the cytoplasmic granules, the border of the vacuoles, in the zona pellucida and finally in the theca interna. There is a gradual increase of this PAS+ve material in the developing ovary reaching a high concentration in the fully mature ovary.

The presence of this PAS+ve material after treatment with malt diastase rules out the possibility of this PAS+ve staining being entirely due to glycogen in the above mentioned structures. The staining after treatment with diastase is slightly less than the staining of untreated sections. This points out that glycogen is also responsible for the staining, but is not the sole cause of the staining.

However, there are other substances that do give a positive staining after oxidation with periodic acid. These are certain lipid substances, and the amino acids, serine, threonine and hydroxylysine, when they occupy a terminal position in the protein chain (Somori, 1952, "Microscopic Histochemistry Principle and Practice," Univ. Press, Chicago, p. 273).

The possibility that the periodic-acid-Schiff reaction is due to lipids can safely be eliminated since lipids are not generally preserved in alcohol dehydrated material. Further the reaction is +ve after extraction of sections with pyridine and methanol/chloroform thus establishing that the staining is due to some material other than lipids.
The staining might be due to any of the above mentioned amino-acids. However the acetylation technique of McManus and Cason (1960, J. Exp. Med., 111, 651 and 1961, Arch. Biochem. Biophys., 94, 293) which is a standard method for "blocking" hydroxyl groups, indicates that the positive Schiff's reaction is due to 1,3 glycol groups of carbohydrates, since the above mentioned sites fail to stain after acetylation. Restoration of these groups by deacetylation (or saponification) in 0.1N KOH is further evidence that the positive Schiff reaction obtained is due to 1,3 glycol groups in carbohydrates and not to the presence of any equivalent amino derivatives of glycol since these latter substances are probably not deacetylated after this treatment (McManus & Cason, 1961, loc. cit. and Hale, 1957, Intern. Rev. Cytol., 8, 194).

It may, therefore, be safely concluded that these sites of PAS-ve material contain 1,3 glycol groups of carbohydrates. It might be that these polysaccharides are reserves for the developing embryo and that they are also the sites of additive synthesis of proteins. The striations of the zona also stain, showing that they contain some polysaccharides. It might be that along these striations only the polysaccharides, synthesised by the theca interna, are transported to the developing ova and they are discharged into the cytoplasm and stored in the granules of the cytoplasm.

The staining with alcian blue shows that it is localised in the granules of the cytoplasm, the border of the vacuoles and theca interna. These granules also stain metachromatically with strong toluidine blue and with azurol A at pH 3.5 and 4.5. The granules show a distinct metachromasia indicating the presence of acid mucopolysaccharides. Staining with AB-PAS shows two distinct types of granules; one the above mentioned and the other close to the periphery just below the zona pellucida.
Since these granules or any of the above mentioned structures do not stain with azure A at pH 1.5 and pH 3.5, it may safely be concluded that sulphated mucopolysaccharides are absent and that the staining with Alcian blue is due to other mucopolysaccharides. In order to determine the nature of the staining material, methylation, both weak and strong, were carried out followed by AB-PAS. It was found that there are two types of granules present in the oocytes. The 1st type like all the other ovarian structures is extinguished by weak methylation and restored after saponification. This is due to the presence of carboxylic groups. The second type of granules are those that are resistant to strong methylation and extinguished by saponification. Lillie, (1968, J. Histochem. Cytochem., 6, 352) has mentioned that certain mucins show this particular type of reaction. The first type of granules and structures contain sialic acid which is responsible for the staining.
PERIODIC-ACID SCHRIFT POSITIVE MATERIAL AND MUCOPOLYSACCHARIDES

Testis : The localization of PAS+ve material was restricted to the walls of testicular tubules, which are made up of fibroblasts and which function as an endocrine organ, as has been pointed out by Marshall & Lofts (1956, Nature, 177, 104) in the testis of the Fim. Mucopolysaccharides were found to be totally absent in the testis of the Lahn; but according to Baillie (1962, Quart. J. Micr., Sc., 103, 385) the basement membranes of the mouse seminiferous tubule contains mucopolysaccharides chondroitin sulphate C which was responsible for the PAS+ve staining in the case of the basement membrane. While studying the testis of the Lahn alcian blue, a specific stain for mucopolysaccharides, was used but it failed to stain any of the testis components, with the exception of the nucleus, which is mostly due to mucoproteins.

Since acid mucopolysaccharides are absent in the testis, all that we have to concentrate on is the neutral polysaccharides. In order to determine the nature of the substances responsible for the PAS staining, the material was submitted to the digestive action of diastase to eliminate the possibility of glycogen being responsible for the staining. The reaction was positive after the action of diastase; this would appear to indicate the presence of potential aldehydes in the polysaccharides. However, other substances are known to give a positive PAS reaction after periodate oxidation. These are certain lipid substances and amino-acids, serine, threonine and hydroxylsine, when they occupy a terminal position in the protein chain (Gomori, 1953, "Morphologic Histochemistry, Principles and Practice," Univ. Chicago Press, p.273). The possibility that the PAS reaction was caused by lipids can be ruled out, since the PAS
reaction was positive after extraction by pyridine and methanol chloroform. Nevertheless, all or any one of the above amino acids could be responsible for the colour produced by the Schiff reagent. However, the acetylation technique of McManus and Cason (1950, J. Exptl. Med., 91, 651 and 1951, Arch. Biochem., Biophys., 21, 293), which is a standard method for "blocking" hydroxyl groups, indicates that the positive Schiff's reaction of the tubule wall is due to 1,2 glycol groups of carbohydrates, since the tubule wall fails to stain after acetylation. Restoration of these groups by deacetylation (saponification) in 0.1M KOH is further evidence that the positive Schiff's reaction obtained is due to 1,2 glycol groups in carbohydrates and not to the presence of any equivalent amino derivatives of glycol since these latter substances are probably not deacetylated after this treatment (McManus & Cason, 1951, loc. cit. and Hale, 1957, Intern. Rev. Cytol., 4, 194). The sections when treated with plain Schiff's reagent gave a-ve reaction showing the absence of naturally occurring aldehydes.

From the study carried out on the testis of the Laban it was found that the PAS material increased from the 1st stage to the 5th stage (fully mature stage). According to Baillie (1962, loc. cit.) with the cessation of growth, there is a progressive aggregation of the carbohydrate-protein complexes, with the concomitant loss of metachromasia and PAS reactivity. In the case of Laban the findings were not like those of Baillie (1962, loc. cit.). The PAS reactivity was the greatest in the 5th stage but this PAS reaction was not due to mucopolysaccharides.
The PAS+ve material was studied in the Laban by the PAS method. In this study of the kidney, it was found that the PAS+ve material was limited only to the brush border of the proximal tubule cells, glomeruli and the interstitial cells. The PAS+ve material went on increasing in all these structures along with the maturing of the fish. It was the maximum in the fully mature fish.

In order to determine the nature of the PAS+ve material the sections were treated with diastase, to see if the staining was due to glycogen. It was found that the staining was less than that of the untreated sections, showing the presence of small quantities of glycogen and the presence of other PAS+ve material.

The sections were then extracted with pyridine and methanol/chloroform to remove only lipid material that might cause the staining with PAS. The possibility of lipid material is a remote possibility, because if any lipids were present in the material, they would be lost during fixation and dehydration in alcohol. The treated sections and their controls were stained by PAS. The results showed that the PAS reaction was not due to lipids. The staining might be due to certain amino-acids. The acetylation technique of McNamara and Gason. (1950, J. Exptl. Med., 31, 651 and 1951 Arch. Biochem. and Biophys., 34, 293) was carried out. The above mentioned structures failed to stain after acetylation. The restoration of the staining after saponification is further evidence that the positive Schiff's reaction obtained is due to 1,3 glycol groups in the
carbohydrates and not due to the presence of any equivalent amino
derivatives of glycol since these latter substances are probably
not deacetylated after this treatment (McManus & Cason, 1951, loc.

The absence of acid mucopolysaccharides in the kidney was
shown by the absence of any reaction when the material was stained
with alcian blue and toluidine blue and with azure A at various pH's.
Longley et al. (1963, Ann. N.Y. Acad. Sci., 104, 423) in their work
on the gold fish found that the mucogenic cells were typically
goblet cells and were irregularly scattered as isolated cells in
the collecting tubules; they were more numerous in the large ducts.
The cells between the goblet cells have fine mucous granules in their
apical cytoplasm. In the case of the toad fish these mucins may be
stained diffusely in the apical region in all ducts large and
small.

In this study of the Labeo, no mucins were noticed, even
as a diffused staining.

Longley et al. (1963 loc. cit.) find mucins in many
reptiles and birds. The proposition that the mucins or mucoid
materials present in the urine could be responsible for the maint-
enance of the colloidal size of the precipitated particles, or in
the formation of pellets as in the case of reptiles and birds, seems
to be quite acceptable when one considers the mode of excretion and
the reabsorption of water and the need to conserve water in the
case of birds and reptiles. But in the case of fish which lives in
water the mucins if present would seem to have some other function.
Longley et al. (1963 loc. cit.) are of the opinion that it is rather difficult to accept this proposition of the part played by mucins in birds and reptiles, for one finds that, it is absent in amphibians, rare in mammals and present in fishes. Only further work will help to elucidate their role in animals.

The distal tubule is known to secrete mucus and Regaud and Péricard (1903, C.R. Soc. de Biol. 58, 679) reported a positive staining of this segment with mucicarmine and with dialysed iron technique for acid mucopolysaccharides. When the kidney of the Laben was stained with Alcian blue which is a specific stain for acid mucopolysaccharides, the distal tubule failed to stain.


The interstitial cells also show the same characteristic as shown by the brush border of the tubule cells. The fact that these cells stain deeply with PAS and are resistant to digestion with malt diastase and various extractions, shows that glycogen and lipids are not responsible for the staining; the cells also do not stain for acid mucopolysaccharides with alcian blue, toluidine blue and Azure A at pH 1.5, 2.5, 3.5 and 4.5. One can safely conclude that interstitial cells contain only neutral mucopolysaccharides.
It is difficult to propose any definite physiological role to the presence of polysaccharides in the brush border and the interstitial cells. It may be that the polysaccharides in the brush border and interstitial cells supply the energy required for synthesis of other compounds and in the brush border they help in the reabsorption of glucose and water in some inter-related way. The staining of the glomerulus is of great significance in the case of human diseases (Jones, 1953, Amer. J. Path., 22, 33 and MeManus, 1950, "Medical Diseases of Kidney", Lea & Febigas, Philadelphia) as also for changes produced in experimental conditions (Bloodworth & Hluswir, 1943, Amer. J. Path., 31, 167). In the present study the glomerulus showed an increase in staining from the 1st stage to the 5th stage (fully mature stage). It is difficult to assign any definite physiological significance to the staining of the glomerulus though possibly the same remarks can be made as in the case of glomerular phospholipids.
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INTERSTITIAL CELLS

Kidney: There are a large number of interstitial cells in the kidney of the Labeo rohita. These interstitial cells are found both in the anterior or head kidney and the posterior part of the kidney or mesonephros. These cells are ovoid or spherical in outline. Their shape, size and number varies with the maturing of the fish as has been found in this study of the Labeo rohita. These interstitial cells have been referred to as the pseudolymphatic tissue by Marshall & Smith (1930 Biol. Bull. 58, 135). They may also be thyroid follicles as shown in the case of satla satia (Ham), Ciromina rebh (Ham) Puntius sarana (Ham) and Labeo rohita (Ham) by Surendra (1962, Curr. Sci., 31(11) 466. The following tests were carried out on the interstitial cells.

Alkaline phosphatase: It was found that alkaline phosphatase was present in these cells and it went on increasing from the 1st to the 5th stage.

Phospholipids were present in large quantities and it increased as the fish matured reaching the maximum in the fully mature fish.

Esterases: The cells do not contain any non-specific esterases.

PAS+ve material: The presence of PAS+ve material was established by using the PAS technique accompanied by digestion with diastase, extraction with pyridine and acetylation and deacetylation. The PAS+ve material turned out to be a polysaccharide which increased from the first to the fifth stage.

Acid Mucopolysaccharide: Alcian blue was tried to ascertain if acid mucopolysaccharides were present. The tests were -ve indicating the absence of acid mucopolysaccharides.
Toludine blue at various concentrations showed no melachromasia.

Azure A. at various jolt's showed complete absence of any melachromatic material.

**Alddehyde Fuchsin**: The interstitial cells stained with aldehyde fuchsin.

**Discussion**: From the presence of all the chemical constituents identified in these cells, one may claim that these cells are secretory in function. It may be on account of this secretory function that the chemical constituents go on increasing during maturation.

From the presence of alkaline phosphatase, it may be concluded that these cells are engaged in active synthesis of other chemical substances. It is also found that phospholipids and the PAS-ve material increases with age. It may be that all these three chemical constituents are interrelated and therefore they all increase together.

The aldehyde fuchsin test was introduced by Gomori (1950 Am. J. Clin. Pathol. 20, 665) as an elastic tissue stain; it was noted to colour some but not all mucins. Abul - Haj & Reinhart (1952 J. Nat. Cancer. Inst. 12, 232) and Scott & Clayton (1959, J. Histochem. Cytochem. 7, 536) observed the polysaccharide character of the AF-positive materials and concluded that the dye reacted with sulphated mucopolysaccharides. Nalmi & Daves (1963,
J. Histochem. Cytochem., 1, 447) noted that AF coloured a variety of connective tissue and epithelial mucins. In general, all mucins that were metachromatized with toluidine blue were coloured also by aldehydes fuchsin but not vice-versa (Moury 1963 Am. N.Y. Acad. Sci., 102, 402). Methylolation, saponification and sulfation experiments and staining of \( ^3 ^5 \) labelled materials gave further evidence of sulphated character of the elements stained by AF (Spicer & Meyer, 1960 Amer. J. Clin. Pathol. 33, 453). Howry (1963 loc. cit.) however believed that AF was not specific for sulphated mucopolysaccharide and that AF could colour substances not even established as carbohydrates.

These cells may be the thyroid follicle cells of Ahiya Surendra (1963 Curr. Sci., 31 (11), 466). He too finds that the thyroid cells contain PAS+ve material which according to him is due to mucoprotein or glycoprotein, since he finds the absence of polysaccharides and lipide. The presence of phospholipide is well established in this study of the Labeo robita, as the tests for phospholipids were carried out after extraction with pyridine. The tests were negative, thus establishing the presence of phospholipide.

If these cells are the thyroid cells then these cells could be expected in the head kidney and a part close to the head kidney. But how can one explained that these cells are also found in posterior most part of the kidney.

One is led to agree with Marshall & Smith (1930 loc. cit.) that these cells form a pseudolymphatic tissue.
Halmi N.S. and Davies J. 1953, J. Histochem. Cytochem., 1, 447.