THE USE OF JAPANESE FROGS IN THE DIAGNOSIS OF PREGNANCY

VI. EXPERIMENTS *IN VITRO* AND HISTOLOGICAL STUDIES ON THE MECHANISM OF SPERMATION IN THE FROG

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According to DE ROBERTIS et al.¹⁾, the release of sperm cells from Sertoli cells under the influence of the pituitary in the toad, *Bufo arenarum*, is accompanied by a marked increase in the size and vacuolization of the Sertoli cells. The vacuoles seem to push the spermatozoa away from the tubule wall. After the release of the spermatozoa, most of the Sertoli cells appear enormously vacuolized, accompanied by the completely irregular apical border, due to the rupture of some of the vacuoles. Hence, the cause of the release of the spermatozoa, which are connected with the testis tubule wall by means of the Sertoli cells, appears to be due to the vacuolization, followed by the rupture and the disintegration of the apical parts of these cells. Besides, they reported that small eosinophilic granules or acidophilic droplets were observed in the Sertoli cells 30 minutes to 1 hour after the injection of three hypophyses of toads.

On the other hand, VAN OORDT et al.²⁾ stated that epinephrine, pregnyl (a HCG preparation) and extracts of frogs' pituitary glands induced spermiation in the grass frog, *Rana temporaria*. These agents probably bring about a fluid uptake by the testis tubules from the surrounding blood capillaries. It is believed that the increase in the size of the large vacuoles at the base of the Sertoli cells near the tubule wall, which press the sperm bundles towards the center of the testis tubule, together with a fluid stream directed towards the efferent duct system, cause the release of the sperm bundles from the Sertoli cells and the subsequent passive transport to the efferent ducts. They observed that the spermiation occurred after an increase in the weight of the testis, which had been placed in hypotonic or frog's Ringer solution, saying that this fact was in favour of the hypothesis to be put forward.

The purpose of this paper is to report the results of experiments *in vitro*, and histological observations on the mechanism of spermiation, using the testes of *Rana nigromaculata* HALLOWELL.

MATERIALS AND METHODS

For the induction of spermiation, HCG (1 MU=12 γ), epinephrine, bovine feces extracts and histamine, were used.

In the experiments *in vitro*, one testis of a frog was soaked in 2 cc of various concentrations of saline solution containing HCG 0.5~1.0 mg, commercial epinephrine hydrochloride solution, diluted 10 times with the medium saline solution—0.1~0.2 cc, histamine 0.0002~2 mg, bovine feces extracts diluted with saline solution, which was a little more hypertonic than 0.769% saline, etc., while the other testis was put into the same concentration of saline solution without the agents. A small piece of the fat tissue, left attached to the testis, was held with a small pincette and the adhering fluids were carefully absorbed on a piece of filter paper for a few seconds. The weight of the testis was measured with a torsion

balance just before putting into the solution, and afterwards the medium solution was left standing for definite periods. Sperm cells in the medium solution were microscopically inspected during the intervals of the weight measuring.

According to KASHIWABARA and KONDO³⁾, the right testes averaged 2.9 mg (0.5~4.5 mg, 3.6%) heavier than the left ones,—in 199 mice. On the contrary, in most of 100 frogs (body weight 16~43 g, average 27.7 g), the left testes (12.6~84.5 mg, average 36.6 mg) averaged 6.3 mg (18.8%) heavier than the right ones (10.5~74.7 mg, average 30.3 mg), in only 9 frogs was the latter heavier than the former.

One testis of a frog was taken and fixed before the injection, while the other one was fixed after the spermiation caused by the agent, in the case of histological studies on the testes of some frogs used in the experiments *in vivo*, to avoid individual differences. No spermiation was observed in three frogs for four hours after the operation for the removal of one testis,—in May, before the breeding season. It may be said that the stress given by this operation does not cause spermiation for at least four hours.

The testes were generally fixed in Carnoy's, Orth's, and Kolster's fluid, or in 10% formalin, and some of them were fixed in a mixture of 2% osmic acid, 1 part, 3% $K_2Cr_2O_7$, 2 parts, and 1% chromic acid, 1 part. The sections of 4~7.5 μ were stained with hematoxylin and eosin, Heidenhain's iron-hematoxylin and eosin, PAS or Unna's method. The sections of the spermiated testis, and the control one, were both stained on the same object-glass to avoid technical errors.

EXPERIMENTS AND OBSERVATIONS

1. Experiments *in vitro*

The experiments *in vitro*, were carried out in the spring, the breeding season of the frog, in the summer, the period of sexual rest, and in the autumn, before hibernation.

Chart 1. Diagram showing the Influence *in vitro* of the Saline Concentration upon the Testis Weight (in May)
The arrows point to the times at which the first sperm cells were observed in the medium.

Chart 2. Diagram showing the Spermiation causing Activity of HCG in the slightly Hypertonic Saline Solution (in November)

Chart 3. Diagram showing the Seasonal Variation (in June & August)

Testis weight increased more extensively, but spermiation did not always occur in summer. Sperm cells released with relatively slight increase of the testis weight in spring

The testes increased in weight, and spermiation occurred, in hypotonic salines and water, but no water uptake nor spermiation was observed, however, in hypertonic saline. These results are the same as those of VAN OORDT et al., already reported (Chart 1).

An adenohypophysis of a frog, ground in 2 cc of 0.645% saline solution, caused an 8% increase in the testis' weight and the spermiation, after thirty minutes, but no increase in the weight nor the spermiation was observed in 0.645% saline only,—in June.

Chart 2 shows that HCG, in a slightly hypertonic saline solution, caused an increase in the testis' weight and the spermiation, but the opposite testis of the same frog, soaked in saline of the same concentration, decreased slightly in weight, and no spermiation occurred.

Almost similar results were obtained with epinephrine. Two testes, in 2 cc of 0.714% saline solution containing 10^{-1} epinephrine hydrochloride solution 0.1 cc, increased in weight 5~10%, and spermiation occurred after 1 hour, while the weights of the control testes decreased slightly

(2~5%), and no spermiation was observed,—in November.

Chart 3 indicates that the water uptake was conspicuous but that the spermiation did not always occur, in summer. On the contrary, spermatozoa were released under a slight increase in the testis' weight,—in spring. It is probable that the testes had already taken up some water under the stimulus of the endogenous spermiation causing factor,—in the breeding season.

One tenth cubic centimetre of 10^{-1} epinephrine hydrochloride solution, even in 2 cc of a slightly hypertonic 0.769% saline solution, caused a 4% weight increase after thirty minutes, and spermiation after one and a half hours; 0.0002~2 mg of histamine in 2 cc of a hypotonic 0.606% saline solution, however, caused no remarkable water uptake nor spermiation, for five hours,—in November.

Bovine feces extract (Extract 5 after Banik's method⁴⁾) 1 cc (equivalent to 2.3 g of feces) in 3 cc of 0.909% saline caused 4% and 7% weight increases in two testes and spermiation in one testis after four hours, while a 2~5% weight decrease, and no spermiation, were observed in two control testes kept in 0.769% saline.

The osmotic pressure of the medium being very important in these experiments, the hematocrit values of the frog's blood in the bovine feces extract, diluted four times with 0.909% saline, and in 0.769% saline were measured, and it was ascertained that the former was slightly more hypertonic than the latter. So it might be said that the bovine feces extracts have a weak spermiation causing potency, *in vitro*.

In the cases of the testes of frogs which were kept in 0.645% saline solution, containing

dibenamine in the concentration of 10^{-3} or 10^{-4} , for two hours, and then epinephrine or HCG being added, it was found that the water uptake and the spermiation were inhibited. This occurred in all the cases of 8 tests using epinephrine, and 3 tests using HCG. The same concentration of nicotine did not inhibit the water uptake nor the spermiation in any of the cases in 5 tests (2 tests using epinephrine, and 3 tests using HCG). These inhibition tests *in vitro*, were performed in October and November.

2. Histological observations

It must first be noted that the histological findings in all tubules were not always equal in the section of a testis, some tubules of the control testes looking somewhat like the weakly reacted tubules of the spermiated testes, some tubules of the latter remaining quite like the former.

It was suggested that a very slow and inconspicuous spermiation process was going on in the intact testes, as RUGH⁵⁾ had already reported, and also, only one sperm cell per one hanging drop has been sometimes observed by us in the cloacal examination, even in summer⁶⁾. On the contrary some tubules of the spermiating testes remained without any change. Owing to such a contradiction, there were some difficulties in the observation of the sections.

On the other hand, it was easy to note the difference between the strongly reacted tubules and the unreacted ones in one section of the spermiated testis. It could also be easily noted under the dissecting microscope (10 times), that the tubules of the spermiated testes had taken up water, the diameters of most tubules being much larger than those of the controls, and there were also spaces in the outer zones of those tubules.

No vacuoles were found in most of the Sertoli cells in the sections of the control testis (Fig. 1), while in the strongly reacted tubules of the spermiated frog, vacuoles were observed in the Sertoli cells, and the cytoplasm of those elements increased in size (Fig. 2). These observations coincide with those of DE ROBERTIS and VAN OORDT.

The cytoplasm of the Sertoli cells in the strongly reacted tubules, and the interstitial tissue of the spermiated testis, fixed in Carnoy's fluid and stained with Heidenhain's iron-hematoxylin and eosin, were more eosinophilic than those of the control testes. Nuclear membranes of the cells in the interstitial tissue, and the Sertoli cells of the spermiated testes, lost their ability to be stained by iron-hematoxylin (Figs. 3 & 4).

The interstitial tissue, Sertoli cells, and germinal cells, showed affinity to pyronine or toluidine blue in the sections of the testes fixed in Carnoy's fluid and stained with pyronine and methyl green, or toluidine blue. The sperm heads showed affinity to both pyronine and methyl green, being stained blue or purple (Fig. 5). The testis tubules of some frogs were diffusely full of the pyronine stainable substance.

The affinity to pyronine or toluidine blue was lost after digestion with ribonuclease; the presence of RNA in those sites being thus ascertained.

RNA in the interstitial tissue, and the reacted tubules of the spermiated testis, decreased or disappeared, both *in vitro* and *in vivo* (Fig. 6).

It is interesting that RNA was found in the interstitial connective tissue of the testes of the frog when it had not been found in the ordinary connective tissue, such as the interlobular connective tissue of the liver (Fig. 7).

Glycogen was also found in the central portion (sperm tail region) of the testis tubules, around the sperm heads, between those and the basement membrane, (mainly in the Sertoli cells) (Figs. 8 & 9), inter or intra germinal cells, and, in some cases, in the lumina of the so-called spermatocysts, lined with a single layer of spermatocytes (Figs. 10 & 11),—from spring to autumn. Droplets of glycogen, of $1\sim2\mu$ diameters, were found in the central portions of the tubules. It is noteworthy that glycogen between the sperm

heads and the basement membrane was more prominently stained by PAS staining after Orth's or Kolster's fixation, than after Carnoy's. Schiff's reaction after oxidation with 4% chromic acid, was much weaker than it was after oxidation with periodic acid solution. It is known that the former oxidant oxidizes some of the vicinal hydroxyl groups to carboxyl or carbon dioxide, making them unavailable for reaction to Schiff's reagent⁷⁾.

The magenta color of glycogen disappeared after digestion with a mixture of 4 parts of saliva and 1 part of the phosphate buffer solution pH 7.0 for 20~60 minutes at 37°C, while glycogen remained in the control section treated with distilled water and the phosphate buffer.

Glycogen in those sites of the testis tubules somewhat decreased in spermiated tubules (Figs. 12 & 13), and the interstitial tissue became thin, and linear PAS staining of the mucopolysaccharides of the basement membrane somewhat shaded off (Figs. 14 & 15).

Some testes put in water containing some india ink, increased in weight rapidly, and spermiation was observed. Histological preparations, after fixing in Carnoy's fluid and staining with Unna's mixture or PAS, included black granules of India ink in the interstitial tissue, and the RNA in the tubules and the interstitial tissue almost disappeared and the glycogen in the reacted tubules also decreased somewhat. The fluid of the surrounding medium might have passed the interstitial tissue and then been taken up by the testis tubules, in the experiments *in vitro* (Figs. 16 & 17).

DISCUSSION

According to VAN OORDT et al., the uptake of fluid by the testis tubules from the blood plasma plays a very prominent part in spermiation. They carried out experiments *in vitro*, in hypo- and hypertonic Ringer solutions. Increase of the testis' weight and spermiation occurred in a hypotonic Ringer solution, no weight increase nor spermiation being observed in a hypertonic Ringer solution. Similar results were obtained in hypo- and hypertonic saline solutions, in my experiments (Chart 1). In some preliminary experiments it was suggested that the isotonic Ringer solution had a slight water uptake promoting influence, but the isotonic saline solution had no or little such influence. Saline solutions were preferred to Ringer solutions to compare the weight of the testis in the medium containing the spermiation causing agent with that of the opposite testis in the medium without the agent.

It is very important that HCG and epinephrine, even in slightly hypertonic saline solutions, caused the increase in testes' weights, and the release of sperm cells, as shown in Chart 2. This phenomenon can not be explained only by the difference in the osmotic pressure. It was suggested that some "chemical reactions" might have occurred in the tissue of the testis. Histological changes brought about by the uptake of water had to be examined, as it was accepted that the water uptake by the testis tubules plays a prominent part in spermiation. Carnoy's fluid was used for the purpose of preserving the water soluble substances in the tissue, and, also, Orth's or Kolster's fluid was used for findings on glycogen and other purposes, no other noticeable findings being observed through the use of the osmic acid mixture fixation.

It is very interesting that RNA was found in the interstitial tissue and in the testis tubules, taking into consideration that RNA has not been observed in the ordinary connective tissue.

Glycogen was found in the central portion (sperm tail region) of the testis tubules, in the Sertoli cells and also inter or intra germinal cells.

RNA in the interstitial tissue, and in the testis tubules, decreased or disappeared in spermiated testes, and the glycogen in the reacted tubules decreased somewhat, both *in vitro*,

and *in vivo*.

Hydrolysis of RNA in the interstitial tissue and the tubules, as one of the above mentioned "chemical reactions", and glycogenolysis in the tubules, as another "chemical reaction", might together play important roles in the uptake of water from the surrounding medium.

Small eosinophilic granules had been observed in the Sertoli cells of the spermiated testis by DE ROBERTIS et al. Eosinophilia of the interstitial tissue and the Sertoli cells of the reacted tubules was found by Carnoy's fixation, Heidenhain's iron-hematoxylin and eosin staining, in my observation. This eosinophilia might have resulted from the disintegration of ribonucleoprotein into RNA and eosinophilic proteins such as protamine or histone.

The decreased basophilia or affinity to pyronine of the interstitial tissue, and the cytoplasm of the Sertoli cells, might depend upon the hydrolysis of RNA into mononucleotides.

Granules of the ink were observed in the interstitial tissue, in the experiments *in vitro*, performed in water containing some India ink, which indicated that the interstitial tissue plays the role of a route for the passage of water from the surrounding medium to the lumina of the tubules.

KIKUCHI⁹⁾ found that the interstitial tissue became thin after the injection of HCG, while on the other hand, KOGA⁹⁾ reported that there was no change in the interstitial tissue a short time after the injection. TAKEUCHI¹⁰⁾ also said that HCG stimulated, directly, the epithelium of the testis tubules, there being no change in the interstitial tissue. The interstitial tissue became thin, in my observation, showing that it was pressed by the swollen tubules. The sharp, linear, PAS positive reddish stain of the mucopolysaccharide of the basement membrane shaded off, somewhat. This is thought to have a correlation with the passage of water through the interstitial tissue to the lumina of the tubules.

According to VAN OORDT et al., the sperm cells are moved passively during the initial stages of spermiation, since their tails are, in most cases, directed towards the efferent duct system, whereas the sperm heads often remain in bundles. This might be true at the very initial stage, but the sperm heads were loosened from their bundles, and directed toward every direction in intensely reacted tubules of the spermiated testes which were fixed from 47 minutes to 4 hours after the injection of the agents *in vivo*, or, immersing in the medium containing the agents *in vitro*, suggesting that the active motility of the sperm cells was accelerated by the monosaccharides yielded by the hydrolysis of RNA and glycogen.

KIKUCHI⁹⁾ reported that the testis tubules swelled, and free sperm cells filled the tubules ten minutes after the injection of a pregnant woman's urine. According to KOGA and KUROI¹¹⁾, the sperm cells were disarranged, and began to detach from the Sertoli cells, after 15 minutes. TAKAGI¹²⁾ also observed that HCG activated the sperm cells in the tubules and caused spermiation in a short time. All these reports are in favour of the above mentioned idea.

ROUGH⁵⁾ reported, in his histological studies on the testes of frogs, that a mechanical stimulus might produce spermiation. On the other hand, taking care to avoid any mechanical stimulus in the removal of the testes, VAN OORDT et al. observed no spermiation of the testis placed in a hypertonic Ringer solution, in spite of measuring the weight of the testis ten times in 5 hours, and in my experiment, also, though the weight of the testis was measured 8 times or more in 5 hours, no spermiation while in a hypertonic saline solution, was observed. Thus spermiation was not so easily brought about by such a careful manipulation as measuring the weight, in their experiments.

Seasonal variation, parallel with that of the sensibility of the frog *in vivo*, already reported¹³⁾, was also observed in the experiments *in vitro*. The testes might have already taken up water from the surrounding blood capillaries under the stimulus of the endogenous

spermiation causing factor, probably secreted from the "purple cells" of the pituitary, as already reported¹⁴⁾, during the breeding season.

Bovine feces extracts showed a weak influence upon the water uptake by the testes, from the medium. BANIK and REINEKE⁴⁾ reported that the purified active substance in cattle feces failed to induce ovulation when injected into virgin doe rabbits, and concluded that the gametokinetic factor differed from the known gonadotrophin and, also, from androgen, in its solubility.

Histamine was inactive in the experiments *in vitro*. It is supposed that histamine, a known stressing agent, stimulates the secretion of epinephrine from the medullary cells of the adrenal glands, and epinephrine stimulates the testes in turn, or activates the adeno-hypophysis by stimulating the hypothalamus, as SAWYER¹⁵⁾ considered in his recent studies on the rabbit.

TAKAGI¹²⁾ observed that 100 mg or 5 mg of dibenamine, injected into a toad, inhibited the spermiation by epinephrine or HCG, and concluding that these spermiation causing agents stimulated the "adrenotropic receptor" in the testis by humoral transmission. The inhibitory influence of dibenamine upon the water uptake and spermiation has also been observed in my experiments, *in vitro*.

On the other hand, it is known that RNA^{16,17)} and glycogen¹⁸⁾ are present in the nerve cells more or less associated with Nissl bodies, and it is also known that the muscle fibers of the impulse-conducting system of the heart contain abundant glycogen. It might be of interest to clarify the correlation among the so-called "adrenotropic receptor", RNA and glycogen, the latter two substances being found fairly abundant in the testis of the frog.

SUMMARY

1. According to VAN OORDT et al., the release of sperm cells took place in a hypotonic Ringer solution, but no spermiation was observed in a hypertonic Ringer solution.

Similar results were obtained with water, hypo- and hypertonic saline solutions (Chart 1).

2. One of the testes of a frog was placed in a saline solution containing the spermiation causing agent, and the other one in a saline solution only, and tests *in vitro*, were carried out.

An adeno-hypophysis of the frog in 2 cc of 0.645% saline solution caused a rapid increase in the testis' weight and spermiation, in the opposite testis, placed in 0.645% saline solution only, a negative result was obtained.

3. It is considered important that HCG and epinephrine in a slightly hypertonic saline solution, caused weight increase and spermiation, while the weight of the opposite testis of the same frog in a saline solution of the same concentration without such agents decreased, and no spermiation took place (Chart 2).

4. Seasonal variation of the fluid uptake by the testis was observed (Chart 3). Rapid spermiation occurred with a slight increase of the testis' weight during the breeding season, indicating that the testis had already taken up water from the surrounding blood capillaries under the stimulus of the endogenous spermiation causing factor which had probably been secreted from the "purple cells" of the adeno-hypophysis, as already reported. On the contrary, spermiation did not always occur in spite of the intense weight increase during the summer,—the period of sexual rest. This seasonal variation showed a parallel relation with that of the sensibility of the frog *in vivo*, as already reported.

5. Histamine caused no water uptake nor spermiation in a slightly hypotonic saline solution.

6. A bovine feces extract, after Banik's method, diluted four times with 0.909% saline, proved to be a little more hypertonic than 0.769% saline solution, and caused a slight water

uptake,—sperm cells being released in a few cases when several tests were made *in vitro*.

7. Dibenamine, of 10^{-3} or 10^{-4} concentration, in an isotonic saline solution, inhibited the water uptake and the spermiation causing activity of epinephrine and HCG, *in vitro*, as reported in the case of the toad, by TAKAGI.

8. Vacuoles in the Sertoli cells, and enlargement of the cytoplasm of these elements, were observed in the sections of the spermiated testes, as DE ROBERTIS et al. and VAN OORDT et al., reported.

9. Small eosinophilic granules were observed in the Sertoli cells of the spermiated testis by DE ROBERTIS et al. Eosinophilia of the interstitial tissue, and the cytoplasm of the Sertoli cells was observed in my observation while both structures remained rather basophilic after Carnoy's fixation, in the control testis.

10. RNA of the interstitial tissue and in the reacted tubules, decreased or disappeared in the spermiated testis. It is interesting that RNA was found in the interstitial connective tissue of the testes of the frog when it had not been found in the ordinary connective tissue.

11. Fluid uptake and spermiation, even in a slightly hypertonic saline solution containing HCG or epinephrine, as stated in 3, can not be explained by only the osmotic pressure. Hydrolysis of RNA in the interstitial tissue, and in the tubules, might be significant as one of the "chemical reactions" supposed to have taken place in the testis. Eosinophilia, observed in the interstitial tissue and Sertoli cells of the spermiated testis, might have resulted from the disintegration of ribonucleoprotein into RNA and eosinophilic proteins, such as protamine or histone.

12. Glycogen, found in the central portion (sperm tail region) of the testis tubules, around the sperm heads, between these and the basement membrane, (mainly in the Sertoli cells) and also intra or inter germinal cells, decreased somewhat in the reacted tubules of the spermiated testes, both *in vitro* and *in vivo*. Glycogenolysis in the tubules may play the role of another "chemical reaction". It was suggested that the monosaccharides, yielded by the hydrolysis of RNA and glycogen, accelerated the motility of the sperm cells.

13. Granules of ink were found in the interstitial tissue of the spermiated testis in the experiments *in vitro*, performed in water containing some India ink, which indicated that the interstitial tissue plays the role as a route for the passage of water from the surrounding medium to the lumina of the tubules.

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妊娠診断に於ける日本産蛙の使用に関する研究

VI. 蛙の排精機構に関する *in vitro* 実験並びに組織学的研究

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DE ROBERTIS 等¹⁾は、ひきがえるに於ける同種下垂体により誘起された排精は支持細胞(セルトリ)の著明な肥大と空胞形成を伴い、此の空胞が精子を精細管腔に押出すと云う。又彼等は同種下垂体 3 ケを静脈注射後 30 分~1 時間に支持細胞内にエオジン好性小顆粒又は酸好性小滴が出現すると云う。

其の後 VAN OORDT 等²⁾は、エピネフリン、HCG 又は蛙の下垂体は蛙の排精を誘起し、此の際精巢の精細管が周囲の毛細血管から水分を吸収するのだらうと云う。組織学的には支持細胞(セルトリ)の底部にある空胞が大きくなり之が精子束を精細管の中心に向って押出し、次で精細管が吸収した水分によって生じた流れで精子は受動的に輸出管系に運ばれると云う。又彼等は *in vitro* で低張リングル液中で精巢重量の増加と排精を観察し、此の事は以上の仮説を支持すると考えた。

以上の事柄に就て、とのさま蛙を用いて、一連の *in vitro* 実験と精巢の組織学的研究を行い所見を得たので報告する。

1. VAN OORDT 等は低張リングル液中では排精が起り、高張リングル液中では起らないと云った。低-高張食塩水及び水を用いて同様の結果を得た(Chart 1)。

2. 同一蛙の精巢の 1 ケを排精誘起物を含む食塩水、反対の精巢を食塩水のみの中に入れて *in vitro* 試験を行った。

蛙下垂体腺葉 1 ケは精巢重量の急速な増加と排精を誘起したが、対照の 0.645% 食塩水のみの中の精

巢では陰性であった。

3. HCG とエピネフリンが弱度の高張液中に於ても精巢重量の増加と排精を起し此の際対照の精巢重量は減少した事は注目に値することである(Chart 2)。

4. *in vitro* に於ても精巢重量増加と排精の関係に於て季節的消長が観察された(Chart 3)。即ち繁殖期に於ては少量の重量増加と共に排精が見られ、精巢は既報¹⁴⁾の下垂体腺葉の所謂紫細胞に依り分泌されると思われる内因的排精因子に依って供試前に既に水分を或程度吸収して居るものと考えられる。之に反し夏期に於ては著しい重量増加が見られるが必ずしも排精を伴わない。之は既報¹³⁾の生体内の感度の季節的消長と一致している。

5. ヒスタミンは弱度の低張食塩水中でも精巢重量の増加並びに排精を起さなかった。

6. BANIK 等⁴⁾に準じて作った牛糞抽出物は、0.769% 食塩水より僅かに高張になる様に食塩水で希釈した場合にも精巢重量の軽度の増加と、数例中 1, 2 例に於ける排精を誘起した。

7. 高木¹²⁾はダイベナミンに依りひきがえるに於けるエピレナミン及 HCG 排精が抑制されることを報じたが、蛙に於て、 10^{-3} 、 10^{-4} 濃度のダイベナミンにより *in vitro* 試験で水分の吸収と排精が抑制された。

8. 排精後の精巢の組織検査に依って、支持細胞(セルトリ)の細胞質内に於ける空胞と細胞体の肥大が見られ、之等の所見は DE ROBERTIS 等及び VAN

OORDT 等の云う所と一致する。

9. DE ROBERTIS 等は排精後の精巢の支持細胞の細胞質中に小さいエオジン好性顆粒が出現したと云うが著者の所見に依れば、排精後の精巢の間質と支持細胞の細胞質はエオジン好性となるに反し対照の精巢では両者共にむしろ塩基好性であった。

10. *in vitro*, *in vivo* を問わず排精後の精巢に於ては間質と精細管内の RNA が減少又は消失した。一般結合組織に見られない RNA が蛙精巢間質で観察された事は興味あることである。

11. 3. に於て述べた所の弱度の高張液中に於ける精巢重量の増加と排精現象は単に浸透圧の関係のみから説明することは不可能である。何等かの化学反応が精巢組織内で起ったことが思考され、此の化学反応の一つとして RNA の加水分解が関係するものと思われる。又間質と支持細胞のエオジン好性の

増加はリボ核たん白が RNA と、プロタミン又はヒストンの様な酸好性たん白に分解したことによるものと思考される。

12. 蛙精巢の精細管の中心部(精子尾部)、精子頭部周囲、精子頭部と基底膜の間(主として支持細胞内)及び精細胞内又は間にはグリコーゲンが証明され、*in vitro*, *in vivo* 何れの場合にも排精後の精巢では弱度の減少の傾向を示した。此の糖原溶解が上記の化学反応の他の一つと思考される。又排精に際し RNA とグリコーゲンの加水分解から生ずる単糖によって精子が活性化される事が推察された。

13. 墨汁を加えた水中に精巢を投入すれば重量の速かな増加と排精が観察され、間質には黒褐色の墨汁の顆粒が見られ、この事は精細管に吸収される水が間質を通過することを示すものと思われる。

EXPLANATION OF PLATE

Fig. 1. Ocul. $\times 20$, obj. $\times 100$ (Oil immersion) (Carnoy fixation, PAS and hematoxylin)

A section of a control testis,—in December. The nuclei of the Sertoli cells are seen adjacent to the basement membrane, and no vacuole is found in the cytoplasm of those elements; a spermatogonium can be found on the right.

Fig. 2. Ocul. $\times 20$, obj. $\times 100$ (Oil immersion) (Carnoy fixation, PAS and hematoxylin)

Three hours after the injection of HCG,—in December. Vacuoles are found between the nuclei of the Sertoli cells and the basement membrane.

Fig. 3. Ocul. $\times 20$, obj. $\times 100$ (Oil immersion) (Carnoy fixation and Heidenhain's iron-hematoxylin and eosin)

The control testis,—in December. Two nuclei of Sertoli cells, whose membranes and nucleoli are prominently stained by iron-hematoxylin, are seen near the basement membrane. The cytoplasm of the Sertoli cells, and the interstitial tissue, are rather more basophilic than those of Fig. 4.

Fig. 4. Ocul. $\times 20$, obj. $\times 100$ (Oil immersion) (Carnoy fixation and Heidenhain's iron-hematoxylin and eosin)

Three hours after injection of HCG,—in December. The nuclear membrane of a Sertoli cell, beneath the sperm heads, lost its stainability; a nucleolus being stained only faintly. The cytoplasm of the Sertoli cells, and the interstitial tissue, became more eosinophilic than those shown in Fig. 3. Some vacuoles are seen in the cytoplasm of the Sertoli cells, between the sperm heads and the basement membrane.

Fig. 5. Ocul. $\times 7$, obj. $\times 100$ (Oil immersion) (Carnoy fixation, pyronine and methyl green stain)

A section of a control testis,—in October. The sperm heads are stained purple with both pyronine and methyl green, and the cytoplasm of the Sertoli cells and germinal cells, and the interstitial tissue, showed affinity to pyronine.

Fig. 6. Ocul. $\times 7$, obj. $\times 100$ (Oil immersion) (Carnoy fixation, pyronine and methyl green stain)

The affinity to pyronine was almost lost from 3 to 5 hours after injection of a bovine feces extract,—in October.

Fig. 7. Ocul. $\times 10$, obj. $\times 40$ (Carnoy fixation, pyronine and methyl green stain)

Comparing with Figs. 5 and 16, where the interstitial tissue of the testis showed affinity to pyronine, the interlobular connective tissue of the liver of the frog was not stained by pyronine, and the parenchymal cells, in the upper half, show affinity to pyronine.

- Fig. 8. Ocul. $\times 10$, obj. $\times 40$ (Orth fixation, PAS and hematoxylin)
PAS reaction of glycogen is found between the sperm heads and the basement membrane, and, also, granules of glycogen are seen in the central part of a testis tubule,—in May.
- Fig. 9. Ocul. $\times 10$, obj. $\times 40$ (Orth fixation, PAS and hematoxylin)
The saliva digested adjacent section of the above. PAS reaction of glycogen disappeared.
- Fig. 10. Magnification as above. (Kolster fixation, PAS and Heidenhain's iron-hematoxylin)
PAS positive glycogen in a spermatocyst was observed,—in August.
- Fig. 11. Magnification as above. (Kolster fixation, PAS and Heidenhain's iron-hematoxylin)
The saliva digested adjacent section of the above. PAS reaction of glycogen in the spermatocyst, disappeared.
- Fig. 12. Ocul. $\times 10$, obj. $\times 10$ (Kolster fixation, PAS and hematoxylin)
A control section, —in August.
- Fig. 13. Ocul. $\times 10$, obj. $\times 10$ (Kolster fixation, PAS and hematoxylin)
One and a half hours after immersion in 2 cc of 0.645% saline solution containing HCG 1 mg. The PAS reaction of glycogen in the tubules was somewhat reduced, comparing with the above.
- Fig. 14. Ocul. $\times 10$, obj. $\times 40$ (Carnoy fixation, PAS and hematoxylin)
PAS positive glycogen is found in the central region of a tubule, and the mucopolysaccharides of the basement membrane are stained sharp, lineally, on the left border,—in May.
- Fig. 15. Ocul. $\times 10$, obj. $\times 40$ (Carnoy fixation, PAS and hematoxylin)
Glycogen in the central part of the tubule disappeared, and sperm heads are loosened from the Sertoli cells and directed in every direction. The sharp, linear PAS reaction of the basement membrane has somewhat shaded off.
- Fig. 16. Ocul. $\times 10$, obj. $\times 40$ (Carnoy fixation, pyronine and methyl green)
The affinity to pyronine in the interstitial tissue, and the tubules was observed,—in November.
- Fig. 17. Ocul. $\times 10$, obj. $\times 40$ (Carnoy fixation pyronine and methyl green)
The affinity to pyronine is almost lost after immersing in water containing some India ink for 90 minutes, and the weight of the testis increased 16%. The granules of the ink are seen in the interstitial tissue and only faintly seen in the outer zones of some tubules.

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