

XX. HIBERNATION STUDIES II.
Histology of the Kidneys During Hibernation.

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In the preceding paper on the hibernation of the frog ** an account is given of the general conduct of the experiments, the histology of which are to be described in the present paper. Twenty frogs from the hibernating series were taken at intervals and the tissues preserved for histological study. In the table accompanying this paper a summary of the animals used is shown. Since profound changes were noted in the histological conditions of the kidneys, that organ was made the subject of a special study.

There is an extensive literature on the physiology and natural history of various animals during the hibernation, included in which literature is a great deal of information relative to the frog while in this condition. Very few observations dealing with the histological changes, however, have been made on any animal during hibernation.

The first observations of importance on the tissues of hibernating animals were made by Gemelli (1906). He found no modification in the nerve-lobe or in the posterior part of the glandular lobe of the pituitary body of hibernating marmots. In the anterior part there were marked changes, especially in the chromophile cells. During the summer these cells were accumulated in big clumps, but during the period of hibernation they were enormously decreased. Shortly after the animal awakened many mitotic figures were found. Changes in the anterior lobe of the pituitary gland consisted in a loss of characteristic topography of the pars anterior, and a shrinkage of both the nuclear and protoplasmic substance of single cells with complete loss of the characteristic histologic condition of the active glandular content affecting the differential staining qualities of the glandular content with acid and basic dyes.

Histological observations on the hypophysis of the woodchuck were made by Cushing and Goetsch in 1913. They confirm in general the findings of Gemelli on the decreases in size and histological changes during hibernation. These changes are ascribed to a period of physiological inactivity, possibly of the entire ductless gland series, but certainly more especially of the pituitary gland, because during the dormant period this structure diminishes in size and shows profound histological changes. Furthermore the deprivation of this gland in the human and experimental animals causes a train of symptoms comparable to those of hibernation.

According to Mann (1926) the theory that hibernation is due to a lack of function of all or any of the ductless glands is not justified. He carried on some experiments on *Spermophilus tridecemlineatus* involving observations on the ductless glands. He quoted Gemelli's observations on the nerve lobe and posterior part of the gland lobe. Mann

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found that the nutrition of the animal is of importance only within very wide limits. A spermophile will not become torpid immediately after eating, and withdrawal of food seems to be a factor producing torpidity. However, it will hibernate even when it has access to a good food supply if the other factors are favorable. He thinks that while changes of temperature are of the greatest importance, they are also effective only within certain wide limits. Very rarely will an animal become torpid at a temperature higher than 24°C. and usually every animal will be lethargic when the temperature gets as low as 5°C. In this study the temperature was found to be the only factor of practical importance, and it was of significance only when carefully controlled.

Mann's study of the tissues of the hibernating spermophile included a number of organs. He showed that there is one slight change which in general is common to most tissues: that of a slight shrinkage of the cell with a decrease in the distinctness of the cell outline and a loss of intensity of the staining reaction. There were marked fatty changes, especially in the liver, and congestion in the spleen. In studying the sex-glands, he found that they undergo a definite seasonal variation but certainly do not play any part as the cause of the hibernating state, nor do they undergo any specific change due to the torpid condition. He was unable to discern any changes in the thyroid of the hibernating animal; the fact that animals in which the thyroids were removed hibernated normally show that these glands are not factors of significance in hibernation. The thymus does not appear to undergo recognizably uniform changes. The islands of Langerhans undergo changes too slight to justify any conclusion in regard to them. The adrenals do not appear to be a specific factor because similar changes were noted in animals whose adrenals had been removed and in the controls, showing that even though the adrenal substance be reduced to the minimum, it did not in any way change the hibernating ability of the animal. Some of the pituitary glands of the hibernating animals showed definite changes, but these changes were not constant.

In the following year, Mann and Della Drips (1917) published their findings on the spleen during hibernation. The spleens from thirty hibernating animals and thirty active animals were studied, the hibernating animals having been dormant from twelve hours to one hundred seventy-five days. The most notable fact in the histology of the organ is the relatively thick trabeculae containing a large amount of smooth muscle. In the gland of the active animal only a small amount of blood is found. The spleen may act as a storehouse for red blood corpuscles in the early stages of hibernation, allowing them to be added to the blood when needed. The blood cells were found to be normal. The authors found that twelve hours after the animal becomes torpid the spleen has a very different appearance, being markedly congested, greatly enlarged, and much darker. Microscopically the organ presents most intense congestion. The sinuses and venous capillaries are distended to their fullest extent with blood. Red corpuscles were found in germinal centers. The spleen reaches its maximum state of congestion within a few days after the animal becomes torpid and maintains this condition until after about forty

days of hibernation. After seventy-five days the amount of blood contained in the spleen is not greatly in excess of that found in active animals. In some animals there seemed to be slight proliferation of connective tissues. These facts are the basis for Mann's conclusion that the statement is unjustified that hibernation is due to lack of function of all or any of the ductless glands.

Sheldon (1924) in a study on the hibernating gland in mammals considered this structure essentially a form of adipose tissue which retains its embryonic character for a more or less indefinite period. He found that this gland in the rat is persistent throughout life, but some of the cells are transformed into ordinary adipose cells. This transformation can be hastened and considerably increased by feeding the animal on a ration rich in fat. A similar but somewhat less extensive formation occurs in the gland as a result of increase in age. The mitochondria found in the cells of the hibernating gland gradually enlarge, and are apparently transformed into fat-droplets. When the rat is deprived of food, the cells of the hibernating gland lose a large percentage of their fat and are much diminished in size. The nuclei, to all appearances, remain practically unchanged by the altered condition of nutrition and growth. It is of interest to note that this gland in the cat is not persistent, but is gradually transposed into ordinary adipose tissue. This transformation is practically complete by the time the animal is nearly grown.

Donaldson (1911), in determining the seasonal changes in the relative weight of the central nervous system of the leopard frog found that it does not change during the year, being constant during hibernation.

Rasmussen (1921), found in his studies of the hypophysis cerebri of the woodchuck that hibernation produces no change in the weight or histological condition of the hypophysis when compared with pre-hibernating glands.

In a series of experiments on winter frogs, van der Heyde (1921), sought to determine the influence of temperature on excretion. He found "that temperature has in reality a tremendous influence on the frog's catabolism. From 0° to about 20° this increase is on'y relatively slight. After 20°, however, the curve rises almost vertically." He shows also that the frog's urine which is absolutely clear at temperatures below 10° becomes colored by a slightly yellowish pigment which appears in the experiments with higher temperatures.

Van der Heyde made no observations on the histological conditions of the kidneys in the winter frogs, but his experiments clearly bear up the observations which are to follow in this paper.

Kater (1927) found that in hibernating frogs mitosis is rarely seen and then only after the animal has been kept in a heated room for one day before fixation. The most striking difference in nuclei of hibernating frogs and those which are active is the great decrease in the amount of chromatin. The cytoplasm loses much of its capacity for staining with basiphilic dyes.

Methods. Frogs which has been kept in the hibernating enclosure varying lengths of time, as indicated on the table, were taken into the laboratory at once after removal from the enclosure and promptly killed; thus no effect of change to higher temperature could

make itself felt upon the tissues to be studied. The first two frogs used were pithed, but all the rest were killed by decapitation and the abdomen opened by a mid ventral incision. The organs were removed as rapidly as possible, one after another, and placed in vials of fixing solution. In two cases Fleming's strong solution was used, but in all others Bouin's picro-formal-acetic was the fixative chosen. It was hoped to secure better cytological preparations by the use of these solutions than commonly result from the more usual histological fixatives.

TABLE I
Temp. O°C. Wt. Chng. Histological Conditions

	Temp. O°C.	Wt.	Chng.	Histological Conditions
	Max - Min	Grams	Pct.	
1. Nov. 18	-----	---	---	
2. Nov. 23 All active	---	---	+ .40	+ 2.00
3. Dec. 8 Sluggish, but roused on handling	6.0	2.7	-4.16	- 9.3
4. Dec. 30 Dormant.	12.2	1.6	+ .67	+ 1.5
5. Jan. 17 Dormant cold, woke up, sluggish	-----	---	-2.53	-11.02
6. Jan. 20 Dormant, slow to wake, very sluggish	7.2	0.0	-4.28	-25.3
7. Jan. 31 Dormant, awoke easier than No. 6.	14.4	0.0	-3.95	-17.6
8. Feb. 2 Awoke, but inactive, reflexes slow	20.0	10.0	-5.21	-18.4
9. Feb. 4 Slightly ac- tive, conscious	21.6	15.5	-7.00	-24.9
10. Feb. 6 Lively	-----	---	-3.90	-14.1
11. Feb. 6 Lively	17.2	12.2	-4.95	-27.8
12. Feb. 5 Lively	-----	---	+1.90	+ 6.7
13. Feb. 12 Quiet, but still awake, sluggish	8.3	5.5	-3.30	- 6.6
14. Feb. 19 Quiet, re- sponded to disturb- ance by "drag- ging" around	4.4	3.8	-4.55	-13.
15. Feb. 23 Mostly quiet, some hop- ped	19.4	4.4	-7.30	-22.1
16. Mar. 6 All quiet (emaciated)	-----	---	-6.42	-24.7
17. Mar. 6 Food in intestine	-----	---	+4.15	+ 9.05
18. Mar. 15 Very active	21.6	12.7	-3.26	-16.5
19. Mar. 20 Active	-----	---	+4.98	+27.5
20. Apr. 10 Wild, normal				

Protoplasm faint, few in-
tertubular spaces.

Tubules drawn apart, larger
nuclei, smaller lumena.

Intertubular spaces larger.

Cytoplasm very granular,
cell walls disappearing.
Spermium in lumena
Epithelium of malpighian
capsule very thin.

Luminal spaces absent cili-
ated border disappearing.
Chromatin staining irregu-
larly.

Nuclei darker. Many mito-
tic figures mostly in meta-
phase about periphery.

Cytoplasm spongy. Some
nuclei wrinkled, others
vesicular. Few mitoses,
large lumena.

Nuclei stain darkly. Very
few mitotic figures. Tubule
walls thickened.

Intertubular space gone.
Nuclear activity. Some
mitoses.

Tubules returned to Nov-
ember condition.

Mitoses again occasionally
present.

The hibernating frogs yielded material from all the more important organs of the body, but only the kidneys were utilized for the present study, the others being stored for future investigation. Paraffin sections varying from 6 to 10 micra were cut in both cross and longitudinal planes of the kidneys. Heidenhain's iron haemotoxylin was the stain depended upon for all results involving cell division.

Normal Histology of the Kidney. Each kidney is a compound tubular gland consisting of a compact bundle of coiled uriniferous tubules, each tubule being equivalent to a nephridium. A tubule begins as a narrow bulb, the Malpighian body, which gradually widens and runs dorsally, forming the neck of the tubule. The neck is lined with short rounded or cuboidal epithelium, each epithelial cell bearing a small number of tiny cilia. The cilia of the cells nearest the capsule are directed toward it, those of the cells further away are in the opposite direction. A second portion of the tube has a coiled course toward the dorsal part of the kidney and then winds toward the ventral surface. It is lined with columnar epithelium, the cells of which possess large distinct nuclei, and are usually covered with a golden-yellow pigment. The third portion corresponds to the narrow limb of Henle's loop; it is lined with ciliated epithelium, similar to that of the neck of the tube. The fourth portion represents the wider limb of Henle's loop. It widens, running in the ventral part of the kidney, and then ascending dorsally to open into a collecting tube. This part is lined with a short, columnar epithelium, which has a free border, consisting of cells with large nuclei and a peculiar arrangement of protoplasm. This protoplasm shows a rod-like structure. The collecting tube, lined with a short polygonial epithelium, runs transversely near the dorsal surface of the kidney and is met by the uriniferous tubules.

Histological Observations on the Kidney during Hibernation. Histological examination of the kidneys of frogs killed during hibernation revealed changes of several kinds in various parts of these organs. These changes include the appearance of spaces between the tubules and the apparent closure of the lumen; the enlargement of nuclei which stained with different intensities and in which the chromatin granules form reticula; the presence among the large vesicular nuclei of others with wrinkled or shrunken nuclear membranes in contrast; the occurrence of mitotic figures in varying proportions in material from different frogs; and some changes in appearance of cytoplasm.

As the frogs remained very active until the first week in December, it would seem that the histological condition of the kidney would not have changed greatly by that time. But in sections of the organ taken from individuals at this time of the year, the anatomical structures stained with different intensities. The malpighian corpuscles were brought out distinctly as they stained more darkly than the surrounding tissues. The loops of the arterial twig and the small amount of connective tissue with which they are held together stain equally darkly. The flattened epithelium of the capsule is clearly distinguishable and appears to be of two layers near the base of the corpuscle as it merges into the neck of the tubule. The protoplasm of

the short rounded epithelium is exceedingly granular; and the nuclei are large and distinct. In portions where columnar epithelium is present it is densely granular and the nuclei are larger and more distinct. The cilia of these cells are short and fine. The lumen of the tubule is not yet closed in frogs of this date, for of course the kidneys are still functioning.

During the month of December, the changes were slight, although the protoplasm lost something of its power to stain deeply. Even though the cytoplasm in the cells of the frogs killed during the month does not appear as granular, the clumps of chromatin in the nuclei are equally as dense as in material previously examined, but the karyoplasm is faintly stained. There is no change in the lumen but the cilia are not as dark as had been noticed in the tissue taken in November. Spaces appear between the tubules as if they were being drawn apart or were shrinking. This condition was at first believed to be partly due to poor fixation, but as this conclusion was not borne out upon further study and the same observations were made on material taken at other times, it was concluded to be a change accompanying structural modifications in the kidneys during the winter months.

On January 31st a frog was killed in which the tubules had drawn further apart, an observation which confirmed the correctness of the technique employed. As the cytoplasm is undergoing shrinkage the nuclei appear larger. There is also, it seems, an actual increase in their size. The lumina of the tubules are smaller.

A change seems to have taken place between January 31st and February 2nd whether climatic conditions could have brought this about is not known, but there was a drop of between 6° and 10° in temperature in these three days. In the frog (VIII) killed on the later date the spaces between the tubules mentioned above have become larger, and the heavy granular condition of the cytoplasm composing the tubules is striking. The cell walls are shrunken and the nuclei are larger, staining more darkly in comparison with the intensity of the protoplasm. Within the lumen there is a coagulum which is globular in structure. In some portions of the tubules the cell wall is not at all distinguishable, for the epithelium is a syncytial layer with regularly arranged, darkly staining nuclei. Areas of nuclei closely crowded together occur in the epithelium giving the appearance of degeneration in these areas of the cortex.

On February 4th, the frog killed (IX) resembled the preceding one. The entire cortex did not show degenerative changes, some of the Malpighian corpuscles retaining their normal appearance but the epithelium of the capsule is very narrow with no trace of a double layer in any portion. The absence of the luminal spaces in the tubules of this frog (IX) is striking. The ciliated edge bordering the lumen appears merely as a line staining a little more deeply than the cells of which it is a part. The nuclei stain with different intensities for in some the chromatin is clumped, these clumps staining more darkly than the karyoplasm of the cell, while in others the entire nucleus stained very deeply, the chromatin granules being diffused through the karyoplasm as heavily staining reticula.

Upon comparing this material with that of frogs previously killed it was noted that the darkly staining nuclei first appeared in frog

VIII although they were infrequent and were only slightly darker than others. In frog IX, the presence of the nuclei with chromatin granules in reticula is quite obvious but the chromatin is not generally distributed as it is in the kidneys of frog XI. In this latter case these nuclei are intensely dark while those in contrast are light as were described above. It is obvious that this condition has gradually taken place and the one mitotic figure present was taken to indicate that the nuclei were preparing for mitosis.

Examination of material of frog XIII, killed February 12th, showed the same general conditions existing, but the darkly staining chromatin reticula are more distinct than any previously described. These are side by side with the ones in which karyoplasm stains faintly and the chromatin is in small clumps. The contrast is very sharp.

Numerous prominent mitotic figures are present throughout, but more numerous along the periphery of the longitudinal section where the cells are not distinguishable, the field being nothing but masses of nuclei. The figures occur so frequently, that several can be counted in one field of the microscope. While the majority of the figures occur in the metaphase plate, several were noticed in the late anaphase stage, and many in the prophase.

As the material taken from this frog is so full of mitotic figures, and the frogs killed afterward had so few, as will be described later, the question arose as to what external condition could have aided in bringing about such phenomena. During the preceding ten days, the maximum temperature was generally between 17° and 20°C. Frog VIII was not dormant but inactive, yet soon after the second of February all grew more and more active until by the sixth they had become very lively. However, there was a drop in temperature from 17.2° on the sixth to 8.3°C. on the twelfth at which time the frogs became quiet. These frogs at this later date were not yet dormant but were inactive. The writers wish to call attention to the fact that this frog had a very large, dark green gall-bladder, and the one fat-body present was very small. It is of interest to note also that except in one case (XII), the frogs had all lost weight between February 2nd and March 6th.

After February 12th, the temperature continued to drop until on the 19th the maximum and minimum temperatures were 4.4°C and 3.8°C, respectively. The frog (XIV) killed on this date was inactive, never attempting to hop in order to escape capture. The cytoplasm of the cells of its kidney is spongy in appearance; the nuclei stained with about the same intensity as before but they vary in size and shape. Some of the nuclear membranes are wrinkled very much like the outside of a raisin, while others are vesicular. It was surprising to note that there were very few mitotic figures present. At first it was thought that there were none but after a careful search two or three were found in each section of the organ.

The material taken from frog XV killed February 23rd is very much like that of frog XIII except for the lack of numerous mitotic figures. The nuclei stained even a little more darkly, but mitotic figures were found only after very close examination in portions where the nuclei were gathered. In the central portion of a section of the

kidney the walls of the tubules are not as thick as they are nearer the periphery.

Attention is called to the fact that this frog had become slightly active perhaps due to a rise of about 13° in temperature. The frogs had begun to feed as some material was in the stomach of this specimen. This frog had practically no blood, but the fat-bodies seem to have an excess within the tissue.

On March 6th two frogs, XVI and XVII, were killed. At the time these were taken from the enclosure, all of the frogs were quiet and under the moss in the cage. The spleen of XVI was very large and dark and there were no fat bodies present. Both XVI and XVII were emaciated although the digestive tracts were crowded with food and there was an abundance of blood. Microscopic study of the kidneys showed them to be in the same general condition. There were practically no intertubular spaces but large cortical areas of crowded nuclei were present throughout the section, as if large syncytia were filled with nuclei. The nuclei in this material seem to be returning to the condition typical of frogs killed in November and December. Generally they stain with the same intensity although a very dark one is found occasionally. In places where the nuclei are numerous they are of different shapes, some being long and narrow while others are small and very wrinkled; others, however, are present which are large and vesicular. A very few mitotic figures were noticed.

Frog XVIII was killed March 15th, at which time the frogs were very active. The intertubular spaces of the kidneys of this specimen were less conspicuous except in the middle portion of the section. The luminal spaces are large but the cilia are not distinct. The nuclei are very interesting as they are of different sizes and shapes, and seem to be arranged differently through the kidney. In the case of the very deeply stained nuclei they are enormous, but do not occur frequently as did those previously noticed. Smaller nuclei are present in syncytial masses out of which apparently new glomeruli are developing.

In frog XIX, killed March 20th, the tubules appeared to be very much like the ones in the frogs killed in November. There were but few spaces between the tubules and those present were small. The luminal spaces were open and a faintly staining material could be seen within. Cilia were distinguishable but stain faintly. The cytoplasm of the cells are not as granular in appearance as in some of those previously described, neither do the cell walls appear very distinct. One or two mitotic figures were found in each section of the kidney, one of these being a distinct metaphase. Some cells stained very dark and some few of the very large ones are present but the mitotic figures are not at all frequent, only one or two being found in each section.

An active *Rana sphenoccephala* was secured from the Canadian River on April 10th. Upon examination of the material from this frog it was found that it was very much like that of frog I. The nuclei were large and generally stained about the same intensity although there were a few throughout which were some darker. No mitotic figures were found but a few nuclei appeared to be preparing

for mitosis. The tubules appeared normal, the luminal space being open with no intertubular cavities present.

Conclusion. This paper is a preliminary report on the histology of the kidney during the hibernation; the conclusions set forth are tentative. The organ undergoes very definite changes during the winter months. The changes visible in material removed from frogs killed after the first of February, present some perplexing problems.

Histological responses due to hibernation appear first in material fixed during the latter part of December and the first of January, and continue gradually to become pronounced until the climax is reached in early spring at which time the structures begin to build up and the tissues return to the condition observed in active animals. Among these tissue responses are the following:

1. Luminal spaces of the tubules tend to become closed. The cilia in portions of the tubules disappear while in others they remain but are distinguishable only as dark lines.

2. Spaces appear between the tubules which seem to enlarge until late spring at which time they are replaced by thickening tubules.

3. The walls of the tubules become narrow as the intertubular spaces appear. This is believed to be due to shrinkage of the cell components thus drawing the tubules apart.

4. The cytoplasm of the cells of the cuboidal epithelium become spongy and loculated while those of the columnar epithelium take on a striated appearance.

5. Beginning in January some of the nuclei enlarge until by the middle of March they are of three sizes: enormous darkly stain-ones; medium sized ones of different shapes with wrinkled nuclear membranes; and small ones of different shapes. They stain with different intensities, those in which the chromatin granules are diffused through the karyoplasm in the reticula staining the darkest.

6. Many of the smallest nuclei are found crowded closely together in areas resembling syncytia. These occur most frequently around the periphery of the section.

7. Numerous mitotic figures occur in the kidney in February but are not frequent in material taken before or after that time, although one or two can be found in each section taken later in the year.

8. The presence of both vesicular and wrinkled nuclei, (the wrinkled ones being numerous) is common in material taken February 19th. This condition persists through the month of March.

10. No change was noticed in the Malpighian body until the early part of March, then the corpuscle seems to shrink in size leaving the space surrounded by the capsule large and almost empty. The appearance of degeneration is suggested.

11. Near these Malpighian bodies which seem to be undergoing retrogression, nuclei are to be observed in rows as if preparing to form new glomeruli.

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