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Microscopic Alterations in Endocrine Glands and Other Organs of Rats Fed the Hepatic Carcinogen p-Dimethylaminoazobenzene

Fenton C. Kelley

MICROSCOPIC ALTERATIONS IN ENDOCRINE GLANDS AND OTHER ORGANS OF RATS FED THE HEPATIC CARCINOGEN p-DIMETHYLAMINOAZOBENZENE

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Fenton C. Kelley

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E. L. Castetter
DEAN

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DATE

Thesis committee

W. J. Eversole
CHAIRMAN

William S. Carter

Arthur L. Campa

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CHAPTER I

INTRODUCTION

It is well known that para-dimethylaminazobenzene (DAB) will cause liver tumors when incorporated into the metabolism of the intact rat (Axelrod and Hofmann, 1953, p. 443; Boyland, 1952, p. 80; Carruthers, 1950, p. 397; Edwards and White, 1941-42, p. 182; Engle, 1952, p. 260; Harris, et al., 1940, p. 623; Kline, et al., 1946, p. 7; Miller and Baumann, 1951, p. 639; Miller, et al., 1949, p. 657; Miller and Miller, 1947, p. 479; Opie, 1944, p. 245; Opie, 1946, p. 104; Price and Laird, 1950, p. 657; Sorof and Cohen, 1951, p. 382; Westerfield, et al., 1950, p. 493; White and Hein,1950, p. 249; Zemecnik, 1952, p. 415). Most of the studies have been concerned with alterations in the chemical composition of the liver and with changes in the involved enzyme systems (Axelrod and Hofmann, 1953, p. 443; Boyland, 1952, p. 80; Carruthers, 1950, p. 397; Hogh-Ligeti, 1947, p. 156; Kensler, et al., 1940, p. 623; Miller and Miller, 1947, p. 479; Opie, 1946, p. 104; Price and Laird, 1953, p. 657; Sorof and Cohen, 1951, p. 382; Westerfield, et al., 1950, p. 493; Zamecnik, 1952, p. 415). Other studies have been concerned with the optimum diet to use for maximum incidence of tumor production with DAB (Harris, 1947, p. 179; Hoch-Ligeti, 1947, p. 153; Kline, et al., 1946, p. 7; Westerfield, et al., 1950, p. 439;

White and Hein, 1950, p. 249); or with the optimum concentration of DAB to use to obtain the highest possible incidence of tumor formation per group of experimental animals (White, etal., 1950, p. 249). It is therefore obvious that the study of carcinogenesis in the livers of rats fed DAB has been undertaken by many investigators from varied fields. However, a survey of the available literature has shown that no studies of the endocrine system and related organs as affected directly or indirectly by this hepatic carcinogen has been undertaken to date. Reports have appeared on some histological changes in the adrenal cortex of rats fed 3'-methyl-4-dimethylaminoazobenzene, a yellow dye related to the one used in this investigation, and such reports will be related to this study in a subsequent section (see Discussion).

Several studies have been made of histological changes and the pathology of developing tumors in the livers of rats fed DAB (Edwards and White, 1941-42, pp. 153-183; Opie, 1944, pp. 231-246) and, therefore, no detailed observations were made herein on the liver itself.

It was the purpose of this investigation to determine whether the feeding of DAB, in addition to inducing liver tumors, alters the microscopic structure of the endocrine system. In addition, a study was made of certain adrenal cortical hormones as possible modifiers of the carcinogenic effects of DAB.

CHAPTER II

METHODS AND MATERIALS

The subjects of the experiment were forty-nine, young, healthy, Sprague-Dawley rats of the same age ± one week. The animals were maintained at a constant temperature of 74±3°F. on diets after the method of Kline and his associates (1946, p. 7). Twelve animals were kept as controls and were maintained on a basal diet. The remaining thirty-seven animals received the basal diet to which 0.06% p-dimethylaminoazobenzene had been added (see Table 1). The animals were maintained on the diets ad libitum for 4 to 9 months.

TABLE 1

INGREDIENTS PER KG. OF BASAL DIET

Ingredients*	Grams	Milligrams
Cerelose	640	
Casein (Vitamin Test)	120	
Salt mixture (Osborne-Mendel)	40	
Corn oil	200	
Thiamin Chloride		3.0
Riboflavin		2.0
Pyridoxine HC_1		2.5
Calcium Pentothenate		7.0
Choline chloride		30.0

* One drop of halibut liver oil per month per rat was given.

Thirteen of the thirty-seven animals on the carcinogenic diet were treated by subcutaneous injection for ten days with adrenal cortical hormones after being fed the carcinogen for 9 months. Ten animals received 5.0 mg. of Cortone (cortisone acetate) per day for 10 days and three animals received 3.0 cc. of adrenal cortical extract per day.

Small groups of animals, excepting those receiving hormone treatment, were sacrificed at half month intervals after being on the diet for 4 months, the last group being sacrificed at 9½ months. After being weighed to the nearest tenth of a mg., the pituitaries, thyroids, thymuses, left adrenals, seminal vesicles, and testes were fixed in Bouin's fluid and preserved in 70% ethyl alcohol. These tissues were later embedded in paraffin and sectioned at 10 microns, with the exception of the pituitaries which were sectioned at 4 microns. Serial sections were taken from the middle of the thyroid, thymus, seminal vesicle, and testis at the greatest diameter of the organ and mounted on slides. The pituitary and adrenal glands were sectioned in their entirety and every tenth section was mounted on slides. Staining of all the sections was accomplished with Krichesky's modification of Mallory's Aniline Blue Collagen Stain after the method of Edwards (1950, p. 247). The sections were studied for histological changes and drawings were

made by Camera Lucida of representative sections and areas of glands that showed the changes to best advantage. Tables 2-4 indicate the histological classifications of the organs from control and experimental animals with the weights and weight ranges of these organs.

CHAPTER III

OBSERVATIONS

A. THE HISTOLOGY OF ORGANS FROM CONTROL ANIMALS ON THE BASAL DIET.

The thymus, pituitary, thyroid, and testis in each of the twelve control animals exhibited a normal histological picture. The adrenal cortex and seminal vesicle of one animal deviated from the normal pattern. The adrenal cortex of this exceptional animal was enlarged and the boundry between the fasciculata and glomerulosa was not as distinct as in the other adrenals of the animals in this group. The seminal vesicle from this animal was degenerate and the size of the organ was significantly smaller than that of the other control animals. The walls of the vesicular lobules were greatly thickened and the lumina contained only traces of colloid.

The adrenal glands and seminal vesicles from the eleven other control animals were normal in all respects.

(For typical histological pictures of the adrenal, thymus, and seminal vesicle consult Plates 1-3.)

B. THE HISTOLOGY OF ORGANS FROM ANIMALS ON THE BASAL DIET TO WHICH p-DIMETHYLAMINOZAOBENZENE WAS ADDED.

1. Adrenal Glands

Of the twenty-four adrenals examined, six showed no deviations from the control animals either in the size of

the gland or in the structure of the cortices and medullae.

Two basic histological deviations were exhibited in the adrenal glands of the eighteen animals that received the hepatic carcinogen in their diet.

The adrenal glands of seven animals were larger than those of the controls and exhibited areas of cellular proliferation both in the zona glomerulosa and in the zona fasciculata (cortical hyperplasia). The cells of the zona fasciculata appeared to be rich in lipoid material and the cytoplasm stained less intensely than in the control glands. These differences can be seen by comparing Plate 4 with Plate 1.

The other basic deviation from the normal adrenal picture was cortical degeneration (see Table 2 and Plate 5), which was observed in eleven animals on the carcinogenic diet. Both the zona glomerulosa and zona fasciculata were involved in this disruption of the normal pattern. There appeared to be a marked decrease in the amount of cytoplasm and the cells were small and densely packed (Plate 5). In some areas of individual glands, the boundary between the the two zones was indistinct, and many pycnotic nuclei were seen in the zona fasciculata. Another evidence of degeneration was the presence of nodules of cellular debris (necrosis). The architecture of the zona reticularis was not modified, but this zone appeared darker than normal

because of the more densely packed nuclei (Plate 5).

In all cases of adrenal cortical deviation, the adrenal medullae were not significantly different from those of the control animals.

2. Thymus Glands

Seventeen of the twenty-four animals on the Carcinogenic diet showed a thymus normal in all respects (Plate 2). Thy thymus glands of the remaining seven animals were degenerate and smaller (Table 3) than those of the controls as can be seen from Plate 6. In some cases glandular lobulation was lost entirely with only collagenous material remaining. They lobules, unlike normal ones, contained few thymocytes.

The six animals that exhibited normal adrenal cortices also had normal thymuses.

Of the seven animals that were observed to have adrenal cortical hyperplasia, two had normal appearing thymuses, while the thymuses of five were degenerate.

Eleven animals evidenced adrenal cortical degeneration and of these only two had degenerate thymuses, the remaining nine thymuses were normal.

3. Seminal Vesicles

Eleven animals on the carcinogenic diet exhibited normal appearing seminal vesicles, the seminal vesicles of two animals were lost in preparation, and the remaining

eleven showed degenerative changes.

As can be seen from Plate 7, the disruption of the normal histological pattern of the seminal vesicles consisted of a marked decrease in the size (Table 4) of the gland as a whole and of the lobules making up the gland. In addition, there were degenerative changes in the glandular pattern. These consisted of a loss of collagen from lumina of the lobules and a collagenous thickening of the lobular wall. There was little or no decrease in the relative amount of secretory epithelium that lined the lumina of the vesicular lobules.

Of the six animals that exhibited normal adrenal cortices, two had degenerate seminal vesicles.

Seven animals had hyperplastic adrenal cortices and five of the these showed degenerate seminal vesicles; the other two animals had normal glands.

Adrenal cortical degeneration was observed in eleven experimental animals. Four of these animals had degenerate seminal vesicles and five animals had normal appearing glands. The seminal vesicles of two animals were lost in preparation.

4. Pituitaries, Thyroids, and Testes

The pituitary gland of each of the animals was carefully examined for any gross histological changes. No changes in the size of the gland as a whole or in the lobes were observed. Neither were any changes observed

in the cellular components of the glands.

Thy thyroids of animals with large hepatomas were likewise carefully examined and no changes in them were observed in either the gross structure or cellular components. As far as could be determined with the methods employed, the thyroid glands from the control animals were not significantly different from the animals on the carcinogenic diet.

The spermatagenic tissue of the testes appeared normal. No conclusions could be reached as to the intertubular tissue since this tissue was lost in the staining procedure due to some unknown fault in the preparation of the tissue for staining.

C. THE HISTOLOGY OF SELECTED ORGANS FROM ANIMALS ON THE CARCINOGENIC DIET THAT RECEIVED CORTICAL HORMONES BY INJECTION.

1. Cortisone Acetate

Ten animals on the carcinogenic diet were injected with cortisone acetate (Cortone) for ten days. All ten animals exhibited extreme adrenal cortical degeneration, which was very similar to the adrenal cortical disruption shown by some of the animals on the carcinogenic diet that did not receive cortical hormones. In most cases the degenerative changes were more extreme. One animal showed widespread vacuolation in the zona fasciculata.

Extreme degeneration of the thymus was likewise observed in all ten animals, which again, was similar to that shown by some of the animals on the carcinogenic diet but not receiving cortical hormones.

Only three of the animals injected with Cortone exhibited degenerative changes in the seminal vesicles. The other seven animals had seminal vesicles that appeared to be essentially normal.

No changes were noted in the pituitary, thyroid, and testis of animals receiving Cortone.

2. Cortical Extract

Cortical extract was administered to three animals on the carcinogenic diet. The adrenals, pituitary bodies, thyroids, thymuses, seminal vesicles, and testes were unchanged when compared histologically to the control animals.

CHAPTER IV

DISCUSSION

The findings reported here demonstrate that feeding the hepatic carcinogen para-dimethylaminoazobenzene (DAB) to male rats causes alterations in the microscopic anatomy of the adrenal cortex, thymus, and seminal vesicles. Although this study did not reveal uniform alterations in these organs, the general histological pattern is similar, if not identical, to that seen in the "General Adaptation Syndrome" (Selye, 1950, pp. 286-348, 452-456, 356-362) during the application of a continuing non-specific stress. The stressor agent in these experiments is believed to be the DAB administered *via* the diet.

According to current hypothesis, prolonged systemic stresses cause hyperplasia of the basophil cells of the anterior pituitary (Selye, 1950, p. 282). Such alterations are believed to cause a shift of pituitary hormone production wherein there is increased production of adrenocorticotrophin (ACTH) and decreased production of gonadotrophin (Selye, 1950, p. 381). An increase in adrenal cortical secretion, due to increased ACTH, results in degeneration of the thymus. Decrease in sex hormone secretion, due to decreased gonadotrophin, results in degeneration of the accessory sex organs. However, an increase in size of the adrenal cortex and degeneration

of the thymus and sex organs is not always evident in organisms subjected to a chronic stress. In a given population, some animals show little or no changes in organ morphology while others exhibit adrenal cortical degeneration with no changes in the thymus or sexual apparatus. It is also possible that a long continued stress eventually leads to a decreased production of ACTH with attendant adrenal cortical degeneration. The results reported here are typical of those found after a prolonged systemic stress. Seven of the experimental rats showed adrenal cortical hyperplasia and most of these had degenerate thymuses and seminal vesicles. Eleven experimental animals had degenerate adrenal cortices and most of these showed no changes in their thymuses and seminal vesicles. Six of the experimental rats exhibited adrenals and thymuses of the same size and histological pattern as those of the control animals. It is possible that the reaction of the pituitary to the stress occured at different time intervals in the various rats and that adrenal cortical hyperplasia was followed by adrenal cortical degeneration. According to such an idea, upon sacrifice, some animals would exhibit adrenal cortical hyperplasia, others adrenal cortical degeneration. The remainder would have normal appearing adrenal cortices. Alternatively, some of the rats may have failed to react to the stress. Some animals may have reacted

by increasing the production of ACTH and others by decreasing this production. Our experiments provide no basis for the selection of one of these hypotheses in preference to the other.

In interpreting these results, it is pertinent to point out that neither the length of time on the diet nor the severity of the liver tumors appeared to modify the histology of the organs studied. Some of the animals with large hepatomas had normal appearing adrenals, thymuses, and seminal vesicles. It is obvious that the regulating factors involved in the apparent hormonal imbalance escape detection at the present time. The pituitary body may be the key endocrine organ involved because it is believed that the state of the internal environment may modify or condition the function of the active pituitary-adrenal system (Skelton, 1950, p. 46). While our studies indicate no changes in the gross morphology of the anterior pituitary, it is possible that functional modifications may have occured without changes in the size of the anterior lobe as a whole.

Alterations in the adrenal cortex of the type reported here after DAB feeding have not been reported previously. Changes have been reported after feeding 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB), a homologue of DAB that is about twice as active as DAB (Miller and

Miller, 1948, p. 148). The liver neoplasms produced by the two carcinogens are similar and probably have a common pathogenesis (Price, et al., 1952, p. 200). Richardson and Borsos-Nachtnebel (1951, p. 399) and Price, et al. (1952, p. 193) reported no pertinent changes in the adrenals of rats fed 3'-Me-DAB, but the former authors did observe atrophy of the thymus. Studies in which 3'-Me-DAB was fed in combination with 20-methylcholanthrene did product adrenal cortical alterations similar to those of the present work and in about the same proportions of rats used (Richardson, et al., 1952, pp. 356-361). Others (Ball and Samuels, 1938, pp. 242-243) have reported adrenal enlargement in intact rats bearing Walker No. 256 tumors, but no enlargement occured in hypophysectomized rats. Tepperman, et al., (1943, p. 377) stated that stimuli such as tumors do not produce adrenal hypertrophy in the hypophysectomized animal. These studies indicate that the function of the pituitary-adrenal axis is modified in tumorous animals. That this axis may be more intimately involved in liver carcinogenesis than previously suspected is suggested by the recent work of Richardson, et al., (1953, pp. 1028-1029) who report that liver tumor formation may be delayed as much as 20 months in hypophysectomized rats fed 3'-Me-DAB. These workers postulated that liver tumor formation is dependent upon a factor (or factors) secreted by the

pituitary. The more recent study of Robertson et al., (1953, p. 779) indicates that ACTH is involved, since hypophysectomized rats fed the carcinogen and injected with ACTH developed liver tumors. Hypophysectomized rats fed the carcinogen and injected with testosterone, desoxycorticosterone acetate (DCA), or cortisone acetate did not develop liver tumors. Since the adrenal cortex is the major target organ of ACTH, one would suspect that cortical secretions are intimately involved in liver carcinogenesis. If such is the case, the action of the essential adrenal cortical factor could not be simulated by administering DCA or cortisone acetate under the conditions of the experiments of Robertson and his co-workers. Although our findings cannot be compared directly with the work done on hypophysectomized animals, it is of some significance that liver tumors were associatedwith adrenal and thymus changes.

It is well known that the injection of cortical hormones will cause adrenal cortical degeneration (Greep and Deane, 1947, p. 424; Ingle, 1938, p. 444; Ingle and Mason, 1938, p. 155; Selye, 1950, p. 301; Selye and Dosne, 1940, p. 167.) and degeneration of the thymus (Ingle, 1938, p. 444; Ingle and Mason, 1938, p. 155; Selye, 1950, p. 469). All of our animals receiving cortisone acetate (Cortone) exhibited degeneration of the adrenal cortex

and thymus. The adrenal cortices, seminal vesicles, and thymuses in animals receiving cortical extract were not significantly modified. It is likely that the cosage of cortical extract used was insufficient to elicit the responses noted with the relatively high doses of Cortone. The injection of Cortone and cortical extract for a ten day period had no apparent effects on tumor growth in the liver.

CHAPTER V

SUMMARY

Forty-nine, young, male, Sprague-Dawley rats of about equal ages were the subjects of this experiment. Twelve animals were kept as controls and maintained on a basal diet. The other thirty-seven animals received the basal diet to which 0.06% p-dimethylaminoazobenzene (DAB), a hepatic carcinogen, had been added. All forty-nine animals were maintained on their respective diets from 4 to 9 months. In addition, thirteen of the animals on the carcinogenic diet received, by subcutaneous injection, cortical hormones after they had been on the diet for 9 months. Ten of these animals were injected with cortisone acetate (Cortone), and three were injected with cortical extract. Small groups of animals, excepting those receiving cortical hormones, were sacrificed at half month intervals beginning with the fourth month and selected tissues were sectioned and examined for histological changes. The adrenals, thymuses, and seminal vesicles were the only organs to exhibit histological deviations from the pattern shown by the control animals.

Seven experimental animals, not receiving cortical hormones, had enlarged adrenal cortices. Five of these animals exhibited degenerate thymuses and seminal vesicles.

Degenerate adrenal cortices were observed in eleven of the experimental animals not receiving cortical hormones. Two of the eleven animals had degenerate thymuses and four showed degeneration of the seminal vesicles.

Normal adrenal cortices and thymuses were exhibited by six experimental animals not receiving cortical hormones; however, two of these animals had degenerate seminal vesicles.

All ten of the experimental animals injected with Cortone had degenerate adrenal cortices and thymuses. Three of the ten had degenerate seminal vesicles.

Organs of the three animals injected with cortical extract were normal in all respects.

It is postulated that the pituitary-adrenal axis is in some way bound up in the mechanism of hepatic carcinogenesis, possibly thru the medium of pituitary adrenocorticotrophin.

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TABLE 2

THE EFFECTS OF p-DIMETHYLAMINOAZOBENZENE

ON THE HISTOLOGY OF THE ADRENAL GLAND

Treatment	Histological Classification	Mean wgt. and wgt. range of adrenal		No. of cases
		mg.	mg/100gm.BW	
Basal diet	Normal	21.9 (18.0-26.8)	6.4 (4.9-7.8)	11*
Basal diet and DAB	Normal	20.6 (19.6-22.0)	7.8 (7.1-8.8)	6
Basal diet and DAB	Cortical hyperplasia	25.8 (22.1-30.6)	11.1 (7.1-14.7)	7
Basal diet and DAB	Degenerate cortex	19.3 (16.8-22.2)	7.8 (5.4-15.8)	10**
Basal diet and DAB plus Cortone	Degenerate cortex	14.5 (12.6-17.8)	6.4 (5.4-8.1)	10
Basal diet and DAB plus cortical extract	Normal	21.1 (20.0-22.6)	7.9 (7.6-8.7)	3

* Twelve glands were studied but one of these was hyperplastic and is not included in this table.

** Eleven glands were studied but the weight of one gland was not recorded.

Treatment	Classification	Histological
Basal diet	Normal	
Basal diet and DAB	Normal	
Basal diet and DAB	Cortical hyperplasia	
Basal diet and DAB	Dysplastic cortex	
Basal diet and DAB plus Cortone	Dysplastic cortex	
Basal diet and DAB plus cortical extract	Normal	

* Twelve glands were examined histologically. Hyperplastic and dysplastic glands were recorded.

** Eleven glands were examined histologically. One gland was not recorded.

TABLE 3

THE EFFECTS OF p-DIMETHYLAMINOAZOBENZENE

ON THE HISTOLOGY OF THE THYMUS GLAND

Treatment	Histological Classification	Mean wgt. and wgt. range of thymus mg.	mg/100gm.BW	No. of cases
Basal diet	Normal	160.0 (38.2-332.0)	48.5 (10.3-72.0)	12
Basal diet and DAB	Normal	122.9 (50.8-229.4)	45.7 (24.1-79.8)	16*
Basal diet and DAB	Degenerate gland	79.9 (8.6-242.0)	29.8 (4.5-78.0)	6**
Basal diet and DAB plus Cortone	Degenerate gland	52.8 (23.8-81.6)	23.0 (12.2-32.0)	10
Basal diet and DAB plus cortical extract	Normal	116.5 (94.4-157.0)	43.7 (34.8-58.5)	3

* Seventeen glands were studied but the weight of one thymus was not recorded.

** Seven glands were studied but weight of one thymus was not recorded.

TABLE 4

THE EFFECTS OF p-DIMETHYLAMINOAZOBENZENE

ON THE HISTOLOGY OF THE SEMINAL VESICLE

Treatment	Histological classification	Mean wgt. and wgt. range of seminal vesicles mg.	mg/100gm.BW	No. of Cases
Basal diet	Normal	816.0 (467.8-1079.0)	249.2 (167-311)	11*
Basal diet and DAB	Normal	706.4 (412.4-1044.0)	252.0 (179-336)	10**
Basal diet and DAB	Degenerate gland	280.0 (105.6-734.0)	117.2 (46.0-384)	11
Basal diet and DAB plus Cortone	Normal	684.5 (298.0-1154.6)	302.6 (144.-473)	7
Basal diet and DAB plus Cortone	Degenerate gland	363.3 (250.0-444.2)	165.3 (96.8-172)	3
Basal diet and DAB plus cortical extract	Normal	720.3 (557.6-839.8)	270.0 (204-323)	3

* Twelve glands were studied but the seminal vesicle of one of the animals on the basal diet was degenerate and is not included in this table.

** Eleven glands were studied but the weight of the seminal vesicles of one animal was not recorded.

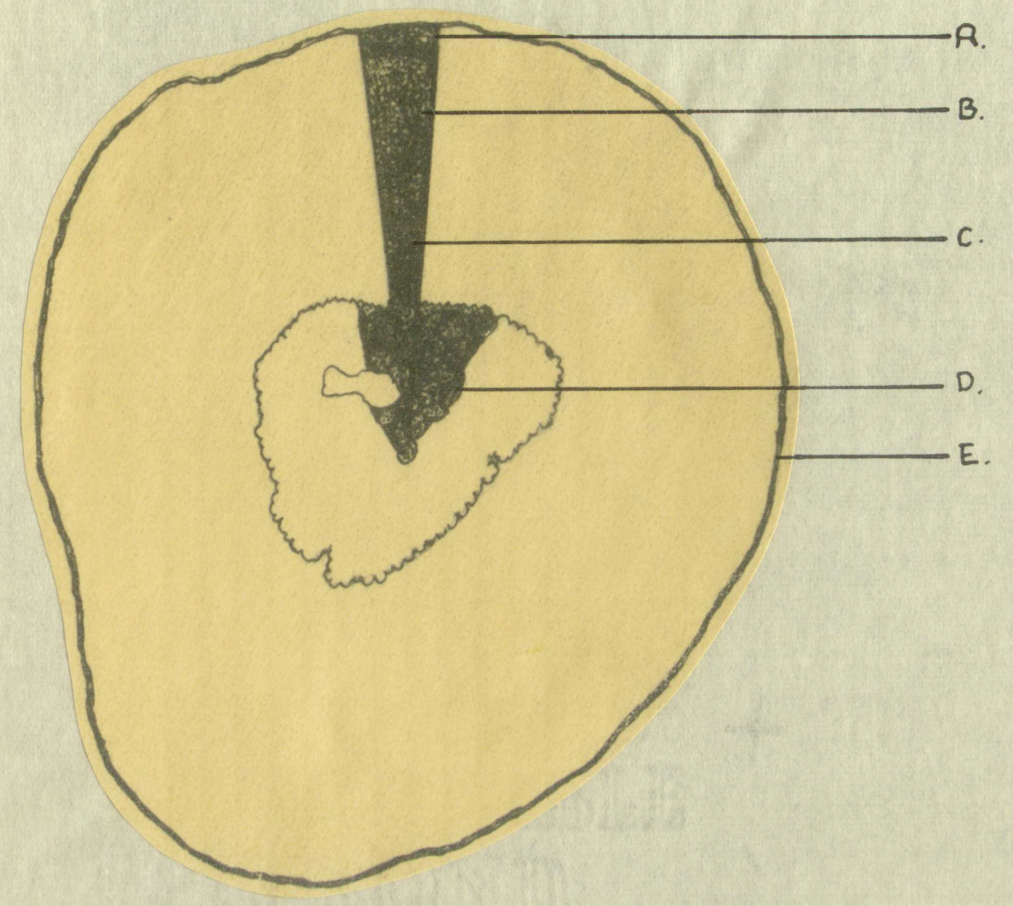


Figure 1

A. Zona glomerulosa
B. Zona fasciculata
C. Zona reticularis
D. Medulla
E. Capsule

Plate 1. Adrenal gland from male rat fed the basal diet for eight months (x20). Weight -- 23.8 mg.

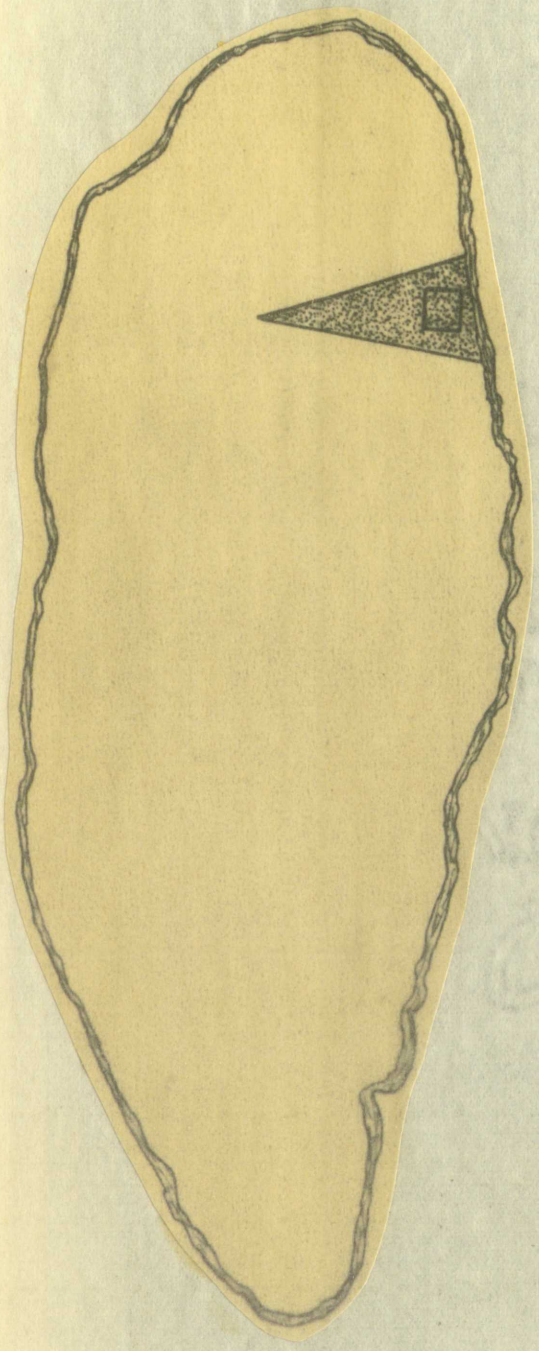


Figure 1

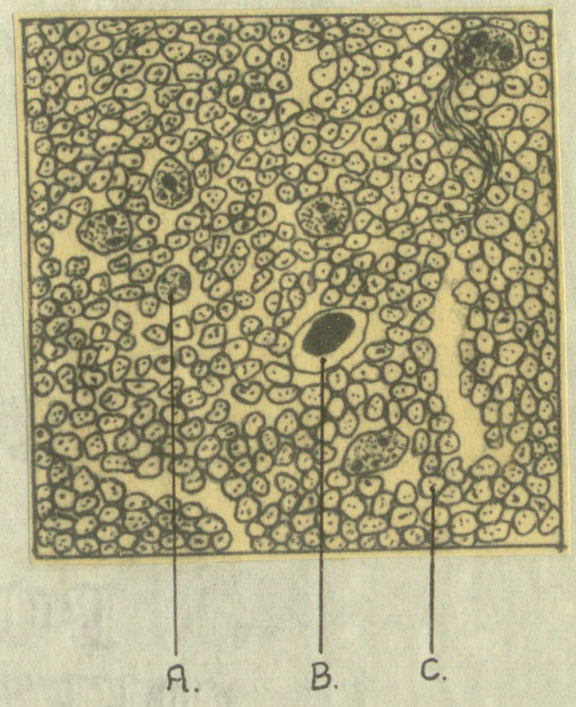
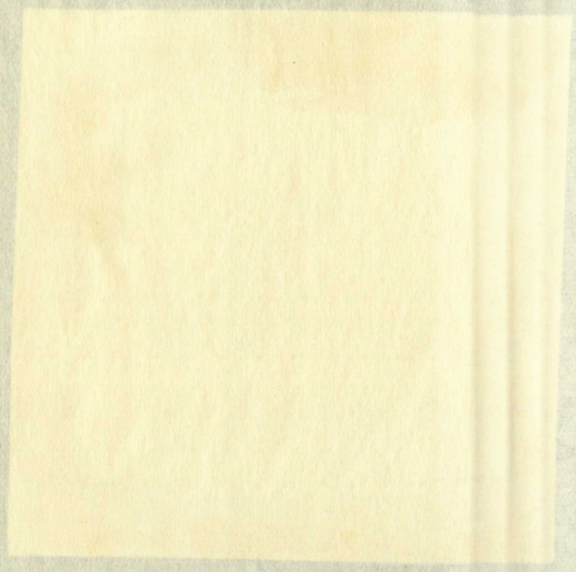
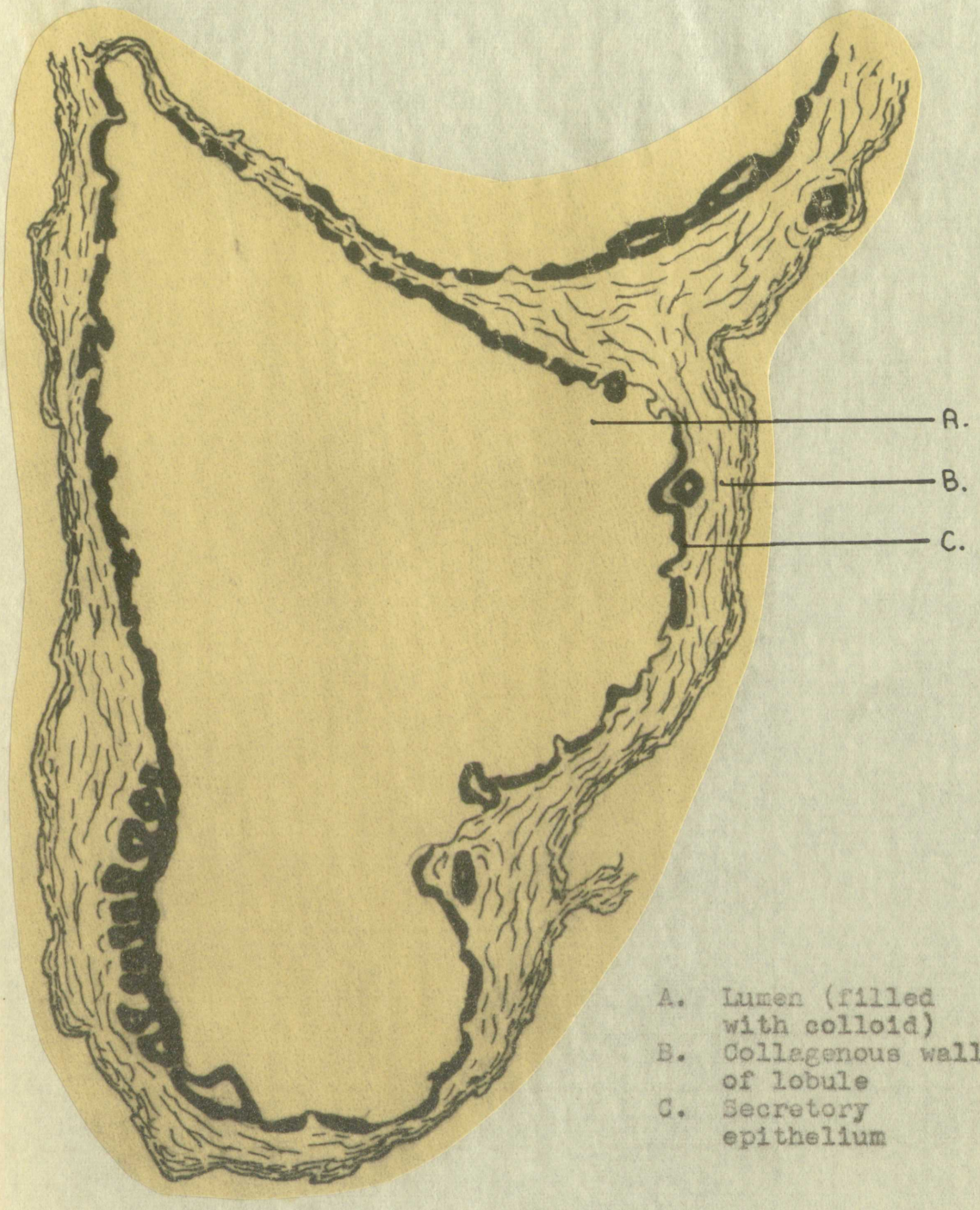


Figure 2

A. Granular leukocyte
B. Hasell's corpuscle
C. Thymocyte (lymphocyte)

Plate 2. Thymus lobule from male rat fed the basal diet for nine months, Fig. 1. To show the contents of the lobule, Fig. 2, (x485) is an enlargement of the small square on Fig. 1, (x50). Weight -- 103.6 mg.





A. Lumen (filled with colloid)
B. Collagenous wall of lobule
C. Secretory epithelium

Figure 1

Plate 3. Lobule from the seminal vesicle of a male rat fed the basal diet for nine months (x50). Weight -- 1010.0 mg.

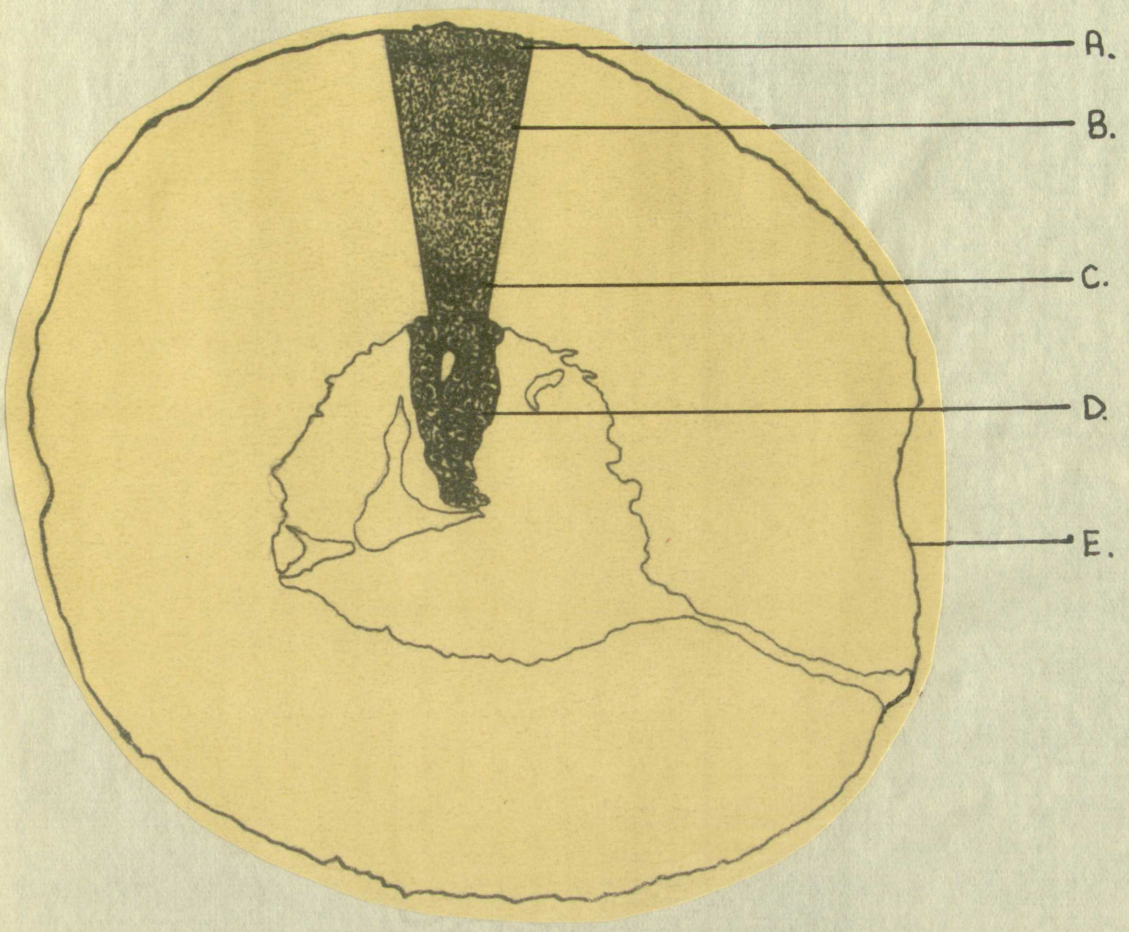


Figure 1

A. Zona glomerulosa
B. Zona fasciculata
C. Zona reticularis
D. Medulla
E. Capsule

Plate 4. Adrenal gland of a male rat fed the basal diet and DAB for five months (x20). Weight -- 30.6 mg. Compare with Plate 1.

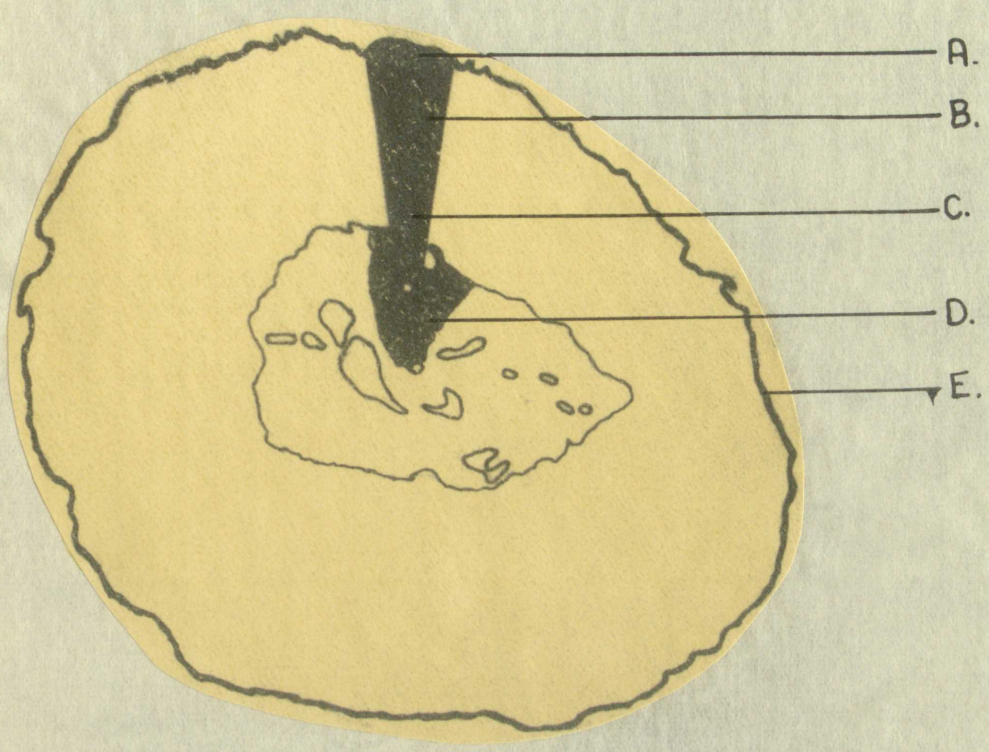


Figure 1

A. Zona glomerulosa
B. Zona fasciculata
C. Zona reticularis
D. Medulla
E. Capsule

Plate 5. Adrenal gland from a male rat fed the basal diet and DAB for six months (x20). Weight-16.8 mg. Compare with Plate 1.

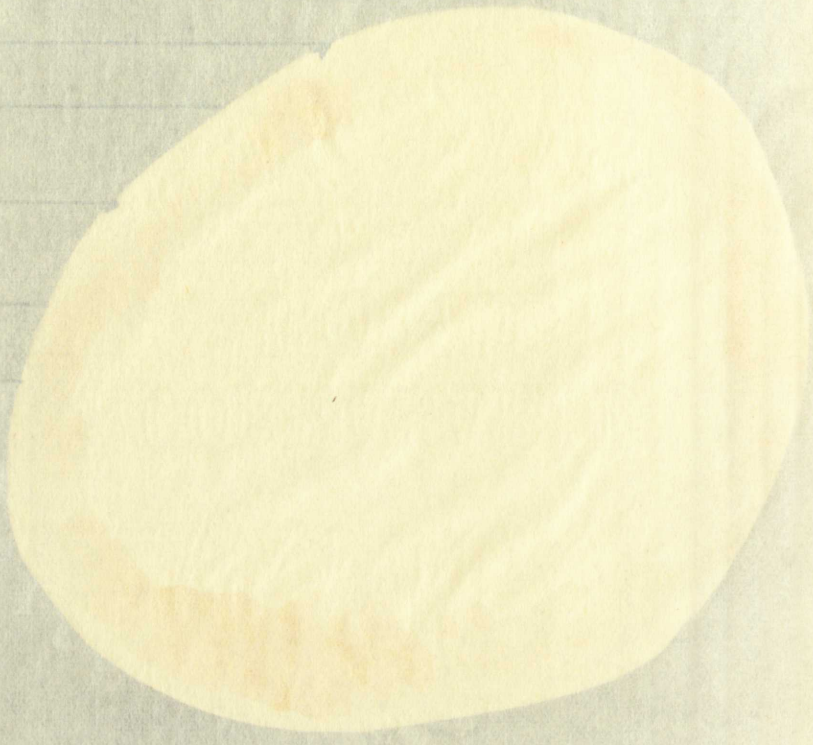


Figure 1

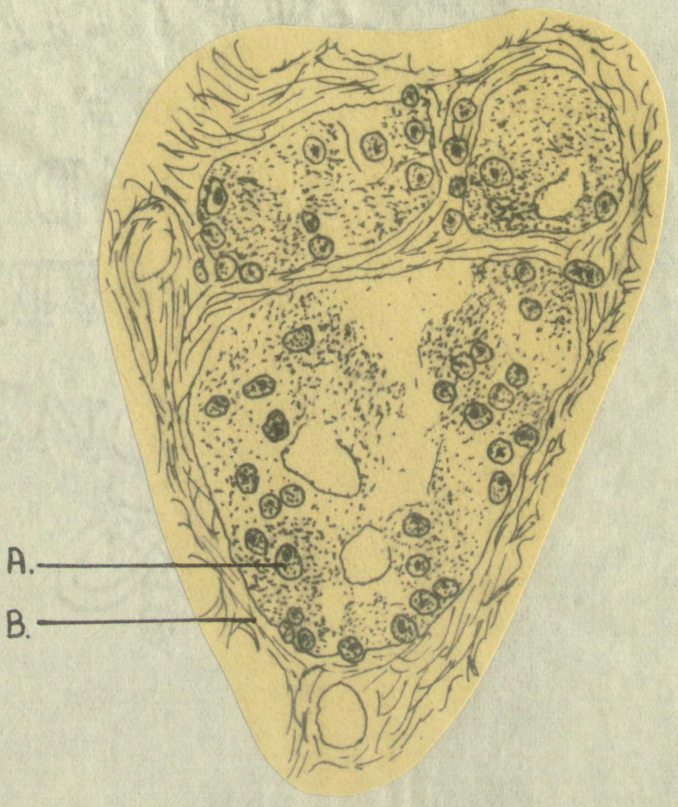
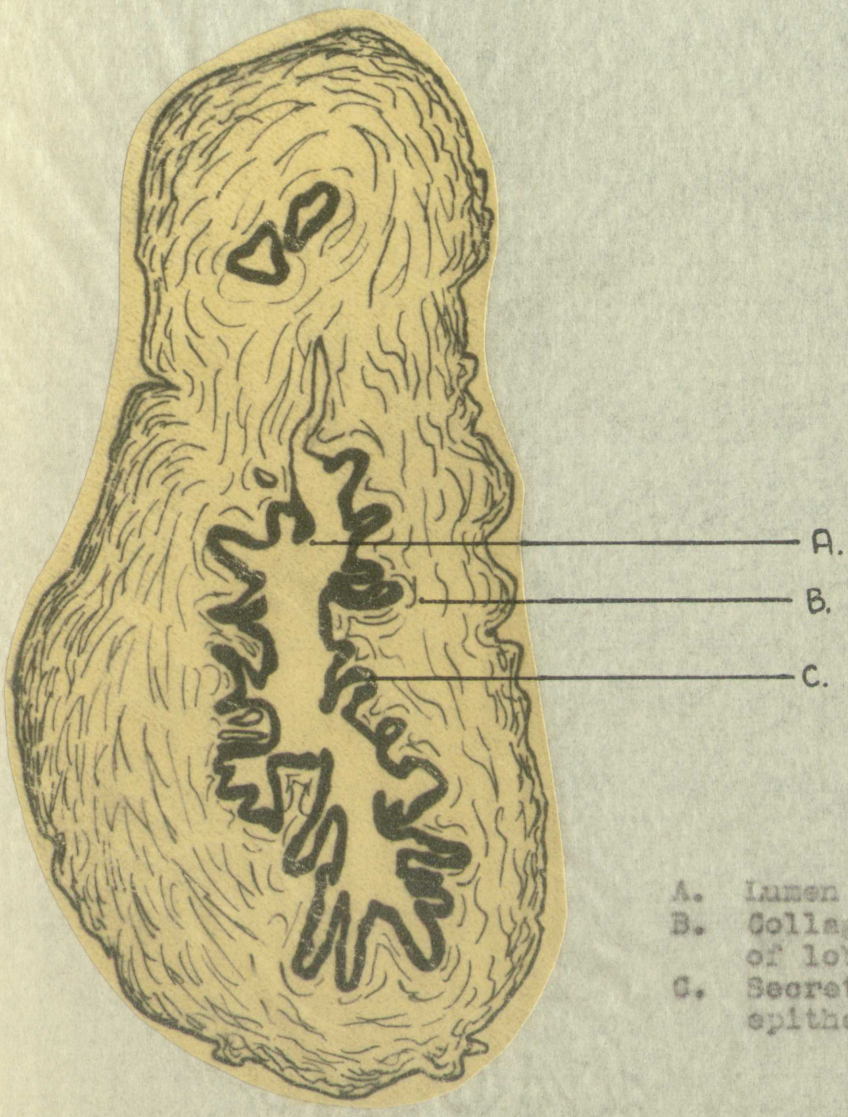


Figure 2

A. Granular leukocyte
B. Collagenous wall of lobule

Plate 6. Thymus lobules from a male rat fed the basal diet and DAB for eight and one half months, Figure 1. To show the contents of the lobules, Figure 2, (x485) is an enlargement of Figure 1 (x50). Weight -- none recorded. Compare with Plate 2.



A. Lumen (no colloid)
B. Collagenous wall of lobule
C. Secretory epithelium

Figure 1

Plate 7. Lobule from the seminal vesicle of a male rat fed the basal diet and DAB for seven and one half months (x50). Weight -- 734.0 mg. Compare with Plate 3.