SPONTANEOUS AMYLOIDOSIS IN DIFFERENTLY GROUPED AND TREATED DBA/2, BALB/c, AND CBA MICE AND THYMUS FIBROSIS IN ESTROGEN-TREATED BALB/c MALES*

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PLATES 51-57

(Received for publication 6 October 1967)

In a previous publication (1) it was reported that sex-segregated grouping of DBA/2, BALB/c, and CBA mice caused an early death in extensive generalized amyloidosis of the males but not the females as compared with mice from groups with one male in a cage with several females. One reason for the amyloid-inducing effect of sex-segregated grouping on male mice may be fighting, but no correlation was found between fighting habits and amyloid development; psychologic stress among members of the social hierarchy in each cage was therefore considered of possible importance (1).

To elucidate further the etiology and pathogenesis of spontaneous amyloidosis in grouped mice, investigations were undertaken on mice of the three strains subjected to the following different groupings: males were caged with males, several males with several females, and one male with several females. Additional groups were all sex segregated and treated as follows; males and females treated with reserpine, castrated males and females, males treated with estradiol benzoate, spayed females treated with chlortestosterone acetate, males and females receiving chlortetracycline, and males and females thymectomized at 1 month of age.

Materials and Methods

Mice of the DBA/2, BALB/c, CBA, and DBA/2 eDe[1] strains were used. The mice were fed chicken pellets and water *ad libitum*, and 6–10 were housed in plastic cages measuring 10 × 15 cm. Thymectomy, oophorectomy, and orchidectomy were performed on 4- to 6-wk-old mice in connection with the final grouping of the mice. Drugs were administered from the same age; reserpine (Serpasil, Ciba, Basel, Switzerland) 1 mg per 100 ml continuously in the drinking water, estradiol benzoate in aqueous suspension (Ovex, Løvens Kemiske, Ballerup, Denmark) 0.5 mg subcutaneously twice a month, chlortestosterone acetate (Caprosem, Løvens Kemiske) 0.25 mg subcutaneously once a month, chlortetracycline (Aureomycin, Lederle Laboratories, Pearl River, N.Y.) 1 mg daily given intraperitoneally for 7 consecutive

* This investigation was supported in part by grants from the Anders Hasselbalchs Fond til Leukæmiens Bekæmpelse, the Carl og Ellen Hertz's Legat til Dansk Læge og Naturvidenskab, and the Danish State Research Foundation.

[1] Breeding litters of these mice were kindly given to us by Dr. M. Deringer, National Cancer Institute, Bethesda, Md.

days each month. A previously described technique was used for electrophoresis on cellulose acetate strips (2). Moribund animals were autopsied and blood was taken for capillary tube hematocrit evaluation and electrophoresis. Tissues were fixed as previously described (1) and stained with hematoxylin-eosin and periodic acid–Schiff (PAS) (3). The degree of spleen and glomerular kidney amyloidosis was estimated on PAS-stained sections and graded from one to six according to Christensen et al. (4). In selected cases Gomori's silver impregnation for reticulin, diastase-PAS, Unna Pappenheim, alkaline Congo red (5) combined with optical polarization examinations (6), thioflavine, toluidin blue, safranin–alcian blue, alcian blue, azur A, aniline blue–chromotrophe 2 R, Giemsa, mucikarmin, van Gieson–Hansen, Lendrum's stain for fibrin, and Mallory PTAH.

RESULTS

Clinical appearance of the mice was good until 6 months of age, when DBA/2 males from the following groups, sex segregated, several males in cage with females, chlortetracycline treated, and thymectomized (see Table I), started developing hunched posture, weight loss, some alopecia, and marks of biting on the back. BALB/c males of similar groups at the same time showed some marks of biting and roughening of the fur. Skin or genital abscesses were not seen. The CBA males were always healthy looking and seldom fought. All other groups of males and females were free of wounds and looked healthy until shortly before death. No pneumonias (1) or other infections were detected in any of the experimental mice.

Survival time of sex-segregated grouped males (our group of reference) was significantly shorter ($P < 0.01$) than that of similarly grouped females of the DBA/2 and BALB/c strains but not of the CBA strain. The same groups of males had shorter mean survival times than males from groups with one male in a cage with several females ($P > 0.05$). Several males in a cage with females had an even shorter survival time (Table I). Reserpine-treated DBA/2 males lived longer than untreated males ($0.01 < P < 0.05$). Castration increased the mean survival time of DBA/2 ($P > 0.05$) and BALB/c ($0.01 < P < 0.05$) males but decreased the survival time of CBA males ($P > 0.05$). Estrogen caused early death with testicular tumors. Chlortetracycline treatment caused some shortening of the mean life span of sex-segregated DBA/2 males ($P > 0.05$). Adult thymectomy had no influence on DBA/2 males whereas it may have resulted in some prolongation of the mean survival time of BALB/c males ($P > 0.05$). Males of the egg-transferred DBA/2 strain had a short survival time when grouped sex segregated both when kept isolated from our conventional DBA/2 mice and when kept in a room with them. The mean survival time of the females of all strains was long, and little affected by the experimental procedure (Table I).

Electrophoresis revealed normal patterns in all mice except for a diffuse hypergammaglobulinemia in a few DBA/2 males.

Macroscopic lesions, including very small thymi and yellow kidneys, were seen in all males except the estrogen-treated males which are described in more

TABLE I

Influence of Different Groupings and Treatments on Survival Time and Spleen Amyloidosis in DBA/2, BALB/c, and CBA Mice

Each group comprises 7–10 mice in one cage

		Grouping and treatment									
		Sex segregated	One male and eight females	Four males and six females	Reserpine	Castrates	Sex hormone	Egg transferred: Isolated	Egg transferred: Not isolated	Chlortetracycline	Thymectomized 1-month-old
Males											
DBA/2	No. of groups	2	14‡	2	2	1		1	3	1	2
	Mean survival time, *months*	12.0 ± 5.5	19.1 ± 3.8	9.1 ± 4.8	17.1 ± 1.7	19.9 ± 3.1		10.2 ± 1.5	9.6 ± 3.1	8.8 ± 1.9	11.3 ± 4.5
	Spleen amyloid*	2.3	0	2.1	0.4	2.0		2.7	2.7	3.4	2.6
BALB/c	No. of groups	4	10‡			2	1 (estrogen)				1
	Mean survival time, *months*	13.8 ± 3.5	21.3 ± 4.7			21.1 ± 3.4	10.3 ± 4.3				18.8 ± 4.0
	Spleen amyloid	1.2	0			2.0	0				0.5
CBA	No. of groups	1‡	5‡			1					
	Mean survival time, *months*	14.6 ± 2.4	18.8 ± 1.6			12.7 ± 0.5					
	Spleen amyloid	2.7	4.0			2.6					
Females											
DBA/2	No. of groups	3	3					1	2	1	1
	Mean survival time, *months*	19.7 ± 3.0	17.2 ± 3.5					19.5 ± 2.3	21.4 ± 0.5	15.6 ± 2.1	17.8 ± 2.6
	Spleen amyloid	0.6	0.4					0	0	1.2	0
BALB/c	No. of groups	3	3			1	1 (androgen)				
	Mean survival time, *months*	20.8 ± 5.4	21.9 ± 3.8			18.1 ± 3.3	20.0 ± 4.3				
	Spleen amyloid	0.1	0			0.4	0.6				
CBA	No. of groups	5‡	3‡								
	Mean survival time, *months*	16.5 ± 2.6	15.8 ± 4.4								
	Spleen amyloid	2.0	2.9								

† The degree of amyloidosis was graded from one to six according to Christensen et al. (4).

‡ Part of these groups were included in a previous publication (1).

detail below. Females always had small thymi, and CBA females had yellow kidneys as well.

Microscopic Lesions.—

Amyloid was found in a majority of the mice, most often in spleen and kidney. In the spleens it was located perifollicularly (Fig. 1) and in the kidneys at the

TABLE II

Staining Reactions of Amyloid, PAS Cells, Thymus Cysts, and Thymus Hyaline in Mice

Staining methods	Amyloid in old, untreated BALB/c, DBA/2, and CBA mice	Old, untreated CBA mice			Estrogen-treated BALB/c males	
		PAS cells near amyloid in spleen, liver, and ovaries	PAS cells in lymph nodes	PAS cells in thymus	Thymus hyaline	Thymus cyst fluid
Periodic acid–Schiff	+	+	+	+	+	+
Diastase-PAS	+	+	+	+	−	+
Alkaline Congo red	+	−	−	−	−	−
Congo red under phased polaroid	Birefringence present	−	−	−	−	−
Thioflavine	+	+	+	+	−	−
Unna Pappenheim	−	−	−	−	−	−
Toluidine blue	+	−	−	−	−	−
Chromotope 2R	+	−	−	−	−	−
Alcian blue	+	−	−	−	−	−
Azur A	+	−	−	−	−	−
Aniline blue	+	−	(+)	(+)	+	+
Safranin	−	−	−	−	−	−
Prussian blue	−	−	−	+	−	−
Giemsa	−	+	+	+	−	(+)
Mucicarmin red	+	(+)	−	−	−	+
Van Gieson–Hansen	−				+	−
Lendrum	−				−	−
Mallory PTAH	−				+	(+)
Gomoris silver impregnation	−				−	−

−, negative reaction.
+, positive reaction.

glomeruli and sometimes also interstitially. Liver amyloid was located between the liver cell cords usually as a clump the size of a liver parenchyma cell (Fig. 3). Amyloid in the ovaries infiltrated the corpora lutea (Figs. 4 and 5). The thyroid gland amyloid was seen interstitially. Thymus, peripheral lymph nodes, and lungs were never affected. The tinctorial properties of the amyloid (Table II) were the same in the different organs and strains of mice. The degree of spleen amyloidosis was fairly constant for mice belonging to the same group

and the amount of glomerular kidney amyloid always closely corresponded to the degree of spleen amyloid. Much amyloid was found in most males including castrates (Table I) although DBA/2 and BALB/c males from cages with one male and several females were usually amyloid-free and reserpine-treated males had only small amounts of amyloid. Females of the DBA/2 and BALB/c strains had little amyloid except in chlortetracycline-treated DBA/2 females. CBA females were very amyloidotic as were the males of this strain.

PAS cells, that is, big cells with strongly PAS-positive cytoplasm without metochromatic granules, were a prominent finding in the spleen, liver, ovaries, lymph nodes, and thymus of CBA mice of all groups; they were less common in DBA/2 mice, and still more infrequent in BALB/c mice. In the spleen (Figs. 1 and 2) PAS cells were located in the perifollicular zone and had abundant, strongly PAS-positive, rather hyaline, cytoplasm. The nuclei were often pycnotic. Amyloid seemed to develop subsequent to the appearance of the PAS cells, and was often located in clumps about the size of a PAS cell. Newly formed amyloid was less PAS positive than the PAS cells but more so than older amyloid (7). In livers PAS cells lined the liver cords and often contained brown granules; these cells were considered Kupffer's cells. The biggest liver PAS cells had strongly PAS-positive hyaline cytoplasm (Fig. 3) and necrotic nuclei. Adjacent to these cells were amyloid clumps of the same size surrounded by a few clumps of brown pigment (Fig. 3). The ovaries contained big PAS cells with granular cytoplasm; amyloid developed in time in the area of these cells (Fig. 4) and PAS-positive granules were found in the amyloid (Fig. 5). Peripheral lymph node sinuses often contained many PAS cells, but amyloid was never observed. The thymus cortex also contained many big cells with vacuolated or granular PAS-positive cytoplasm without brown granules. These cells were numerous in all strains, though most common in the CBA's, and the number was estimated to be roughly the same in mice of all groups, excluding the estrogen-treated mice. Thymus amyloid was never detectable. All thymi were highly depleted of cortical lymphocytes, especially the androgen-treated spayed females, followed by the males of the various groups, while the females had most lymphocytes left. A few small cysts containing PAS-positive fluid with occasional small basophilic clumps were found in many thymi. Different staining procedures (Table II) failed to demonstrate different tinctorial properties of PAS cells from the different organs. However, some of the clumps of homogeneous material in liver sinuses which stained with the amyloid stains may have been located in living Kupffer's cells.

Other microscopic findings were occasional mammary tumors and benign pulmonary adenomas. Leukemia was found in 10% of BALB/c females and less frequently in other groups. Neither mean survival time nor degree of spleen amyloidosis was much altered if the leukemic mice were excluded from the groups.

Estrogen-Treated BALB/c Males—Of the estrogen-treated BALB/c males four were sacrificed when very ill, at only 6 months of age. These four mice exhibited spleen atrophy with depletion of lymphocytes, and thymus atrophy with few lymphocytes, few PAS cells, and many cysts. The testes showed a hyperplasia of deeply PAS-positive Leydig's cells (Fig. 6). The remaining five mice lived until 13 months of age when one or both testes of all mice were enlarged by tumors of interstitial cells showing varying degrees of PAS positivity (Fig. 7). An extreme depletion of lymphocytes was noticed in the atrophic spleens (Fig. 8) which were free of PAS cells. Amyloid was not found in spleens or any other organ in contrast to 10 untreated sex-segregated grouped BALB/c males killed at 10 months of age with a mean degree of spleen amyloidosis of grade 1. Peripheral lymph nodes of the estrogen-treated males were depleted of lymphocytes but contained many big moderately PAS-positive cells in sinuses (Fig. 9), and one node exhibited a small fibrotic area (Fig. 10). Lung, kidney, liver, and thyroid gland looked normal. The thymi were very small and showed only a few remaining lymphocytes (Fig. 11) many of which were necrotic, and very few PAS cells. Large areas contained a homogeneous somewhat striated material (Fig. 12) staining positive with van Gieson-Hansen (Table II). Lymphocytes and PAS cells were absent from these areas. Epithelioid cells and many irregular spindle-shaped cells resembling fibroblasts were, however, found. The remainder of the thymi was dominated by big irregular cysts lined by flat cells without visible brush borders and containing PAS-positive fluid in which many necrotic nuclei and smaller basophilic clumps were found (Fig. 13). Between the cysts were found lymphocytes, epithelioid cells, and some fibroblasts.

DISCUSSION

The shorter mean survival time, the striking clinical symptoms, and the more extensive amyloidosis of sex-segregated male mice as compared with sex-segregated females and with males from cages with one male among many females are in accordance with our previous observations and with the notion that amyloidosis is the prime cause of their early death (1). That several males living in a cage with females succumbed as rapidly as sex-segregated grouped males demonstrates that it is the exposure to other males and not the sexual isolation which induces the amyloidosis. That reserpine in doses which do not inhibit the androgen secretion (8) but exert a tranquilizing effect, can nearly eliminate the differences in survival time and amyloid development between males exposed to other males and males exposed to females is in accordance with the presumption (1) that amyloid development may be related to psychologic pressure among male members of the social hierarchy in each cage (9–11). Involvement of testicular hormones in the amyloid development of sex-segregated grouped males is apparent from the late death in amyloidosis

of castrated males. A direct stimulation of the amyloid-producing tissues by androgen is improbable as our androgenized spayed females had a long survival time and nearly the same small amount of amyloid as spayed females. Androgen may instead be a prerequisite for a stress reaction in the males. An evaluation of the lacking amyloid development in our estrogen-treated males is complicated by the probable enhanced production of endogeneous androgen by the hyperplastic Leydig's cells and interstitial cell tumors (12). A specific inhibition of amyloid production by estrogen seems unlikely as injection of estrogen has been reported to cause amyloidosis in spayed C3H females (13), however, the depletion of lymphocytes may have prevented amyloid development (14). An inhibition of a psychologic stress reaction by estrogen is, however, equally conceivable. Infections characteristic of our mouse colony cannot account for the effects of grouping males with males as identical lesions were found in egg-transferred mice from another colony and their offspring born here. That infections, including infected skin wounds from fighting, should be responsible for the amyloid development is made less probable by the finding that amyloid developed rapidly in the nonfighting CBA males (1), that the long-living castrates which bore no signs of biting were much more amyloidotic than the long-living reserpine-treated or "isolated" males which also were free of skin wounds, that aureomycin did not prevent amyloid development in grouped males, and that thymectomy which lowers the immunologic capacity (15) did not shorten the survival time. If thymectomy retarded the spontaneous amyloid development in sex-segregated grouped male mice as indicated by the prolonged survival time of thymectomized BALB/c males, it could be due to the immunologic alterations (15, 16) or to the ablation of thymus' influence on the gonads (17, 18).

The location of PAS cells near amyloid and the apparent conversion of dead PAS cells in spleen, liver, and probably ovary to amyloid clumps indicated for the first time a local origin of the amyloid from PAS cells in spontaneous amyloidosis as previously demonstrated for casein-induced mouse amyloidosis by histochemical studies (19) and by electron microscopy (20, 21) of amyloid in spleen and liver. The staining reactions of the PAS cells and amyloid observed were also similar to those in casein-induced amyloidosis (7, 22). If the PAS cells in lymph nodes are similar to those in spleen, local factors will have to be held responsible for the lacking development of amyloid in this organ.

The thymus lesions found in the estrogen-treated BALB/c males is probably the result of both injected estrogen and endogenous androgen (12). The lymphocyte-depleting effect of estrogens and androgens (23) is known; long-term estrogen treatment is, however, also reported to increase the mitotic activity of thymus lymphocytes (24). It is, therefore, surprising to find a depletion of thymic PAS cells which probably stimulates lymphocyte mitosis (25). Endogenous androgen might deplete thymus of PAS cells, but the an-

drogenized spayed females were not similarly depleted of thymus PAS cells. Induction of thymus cysts in estrogen-treated mice has been described previously (26, 27). The many dead nuclei and basophilic clumps found in our cysts may be detached from lining cells or reflect an exaggerated physiologic process as small clumps occasionally were found in untreated mice. The very large cysts in the (exhausted?) thymus could be explained if the lining cells were producing the lymphocyte-stimulating hormone (28) in response to necrosis of the lymphocytes. The very prominent intralobular development of fibrous structures, which staining reactions indicate are collagen (29), have to our knowledge not been reported previously in mice. Both injected estrogen and endogenous androgen may have been important for its development as simultaneous injection of these hormones causes prominent thymus fibrosis in rats (30). A leakage of cyst content through the thymus barrier (28) is hardly the cause of fibrosis as collagen was also found in a lymph node. Continuous lysis of lymphocytes faster than the PAS-positive macrophages (25) can engulf them may have provided the irritant causing thymus fibrosis. A more favorable ratio between dying lymphocytes and macrophages in the lymph nodes may explain the paucity of fibrosis there.

SUMMARY

Sex-segregated grouping of DBA/2, BALB/c, and CBA males caused rapid amyloid development and early death as compared with segregated grouped females or with males living individually in cages with several females. Grouping of several males in a cage with females also caused early death in amyloidosis indicating that the exposure of males to males and not the sexual isolation was important for the amyloid development. Both reserpine treatment and castration prolonged the survival time of sex-segregated grouped males. Estrogen treatment retarded amyloid development in sex-segregated males while spayed and androgen-treated spayed females showed only small amounts of amyloid. Treatment with chlortetracycline did not prevent amyloid development in grouped males. Thymectomy of sex-segregated males at 1 month of age gave inconclusive evidence of a prolongation of survival time. Egg-transferred DBA/2 mice reacted as conventional DBA/2 mice when grouped by sex segregation. Cells with abundant PAS-positive cytoplasm were found in the spleen, liver, and ovaries of mice of all strains but most prominently in CBA mice. Evidence for a direct conversion of these cells to amyloid was found. Estrogen-treated BALB/c males developed testicular tumors and thymus alterations including necrosis and depletion of lymphocytes, depletion of PAS cells, formation of large cysts containing necrotic nuclei, and intralobular fibrosis.

We are grateful to civil engineer J. U. Dalgård for performing the statistical calculations, to Mr. Bent Børgesen for the preparation of the photomicrographs, and to Mrs. Sanne Holmberg for performing the special stainings.

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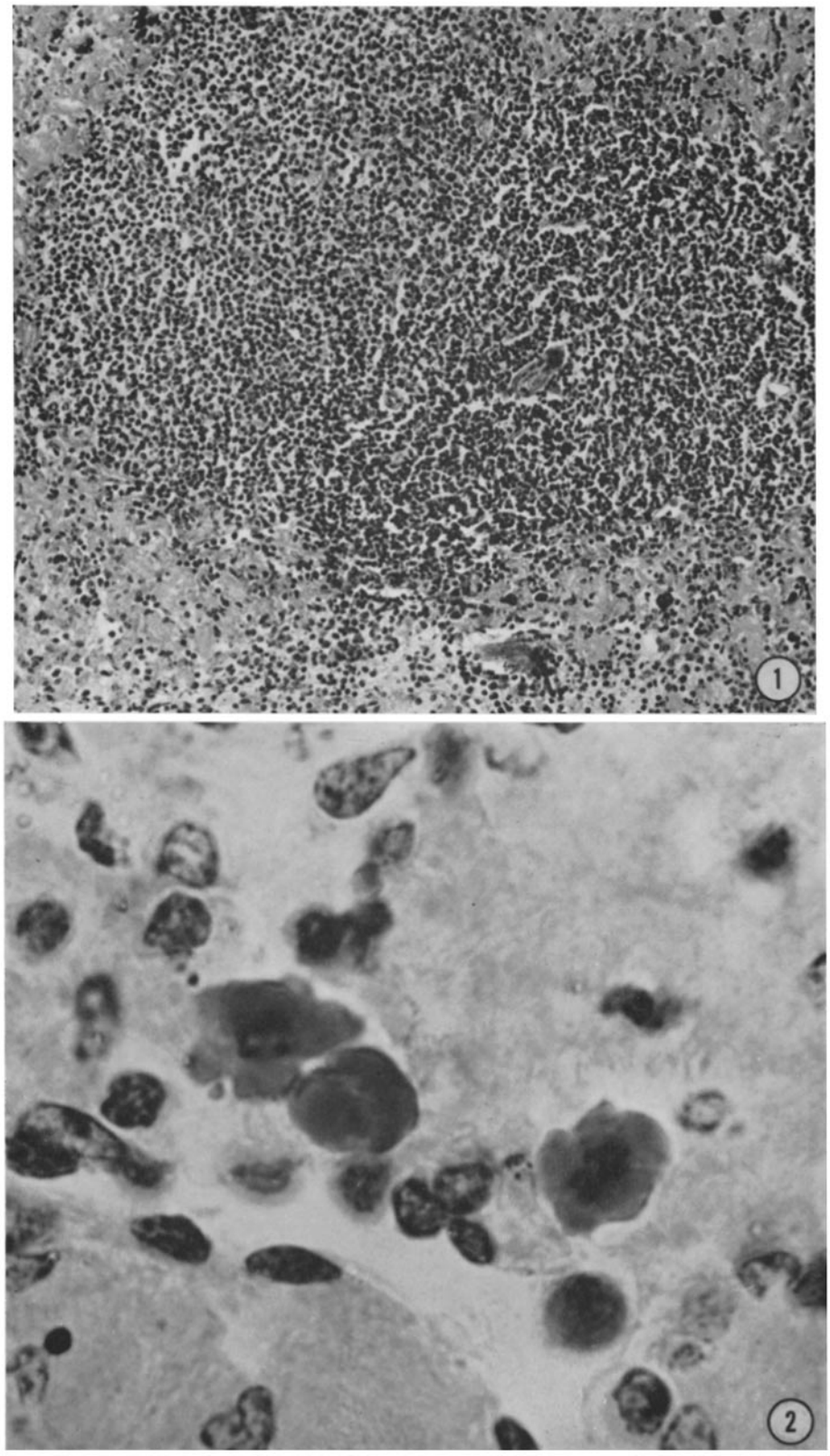
EXPLANATION OF PLATES

PLATE 51

FIG. 1. Section of spleen with amyloidosis grade III from 15-month-old untreated CBA female mouse. Amyloid and PAS cells in the perifollicular zone. PAS technique. × 200.

FIG. 2. PAS cells from the spleen of same mouse as in Fig. 1 with deeply PAS-positive hyaline cytoplasm and necrotic nuclei. Disintegrating PAS cell (left) seems to fade out in adjacent amyloid in the amyloid necrotic nuclei (upper right). PAS technique. × 2000.

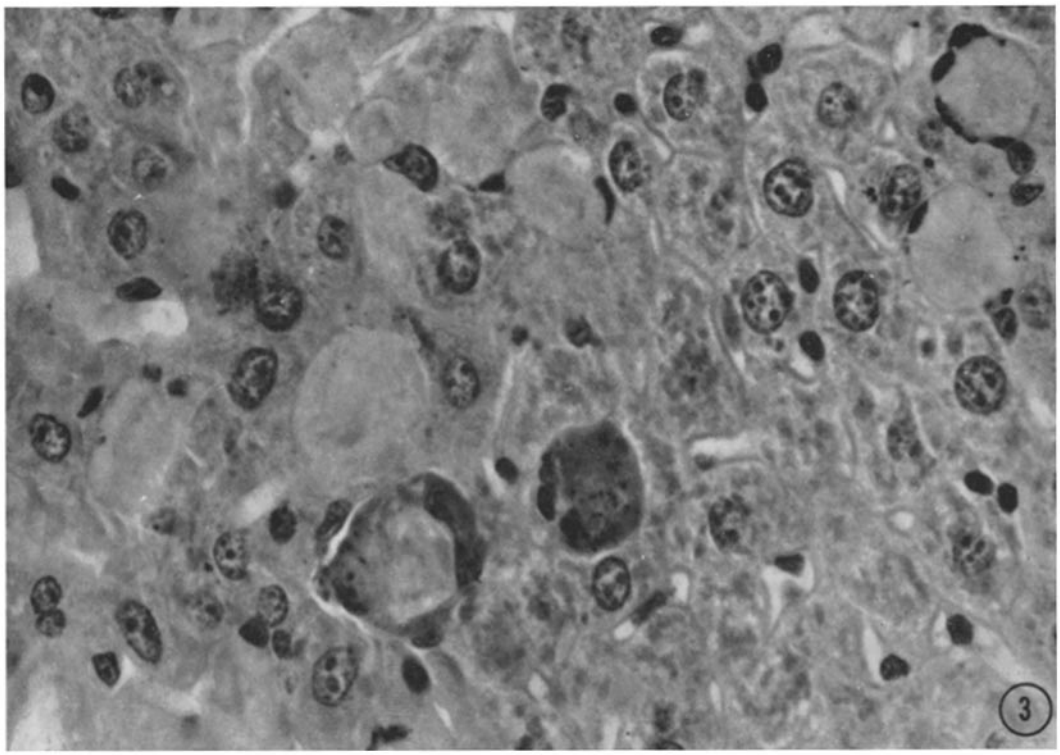


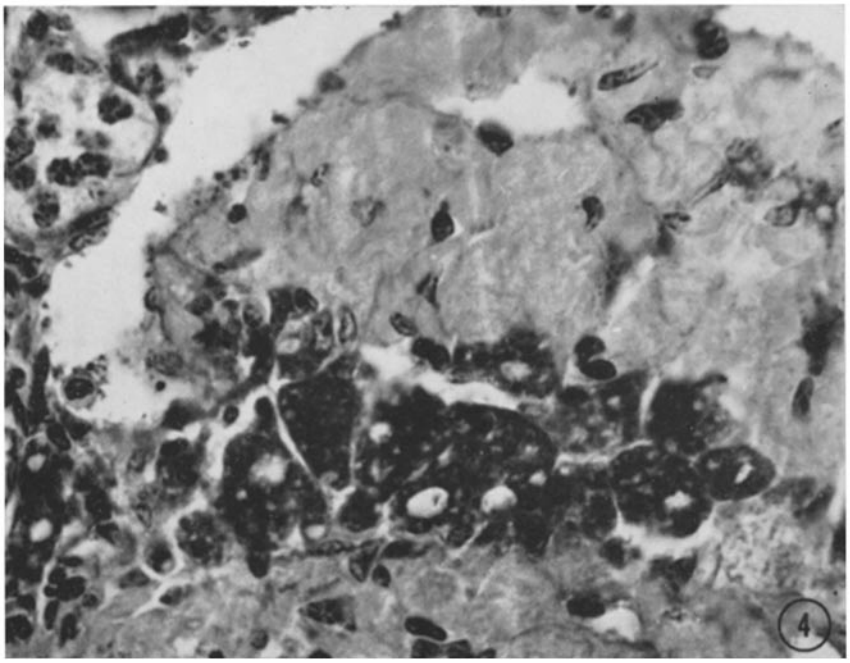
(Ebbesen: Spontaneous amyloidosis and thymus fibrosis)

PLATE 52

FIG. 3. Amyloidotic liver from 15-month-old untreated CBA female mouse. A big Kupffer's cell is seen in a sinus (center) with deeply PAS-positive cytoplasm containing pigment granules and (left) also a Kupffer's cell with beginning hyalinization of the cytoplasm which still contains a few granules. Note the similarity in size of the Kupffer's cells and the amyloid clumps in sinuses. PAS technique. × 840.

FIG. 4. Ovary from 17-month-old untreated CBA female mouse. Most of the ovary has converted to amyloid, in close connection with which are big cells with PAS-positive cytoplasm. The PAS-positive cytoplasm seems to merge into the amyloid substance in which some small PAS granules are preserved. PAS technique. × 525.

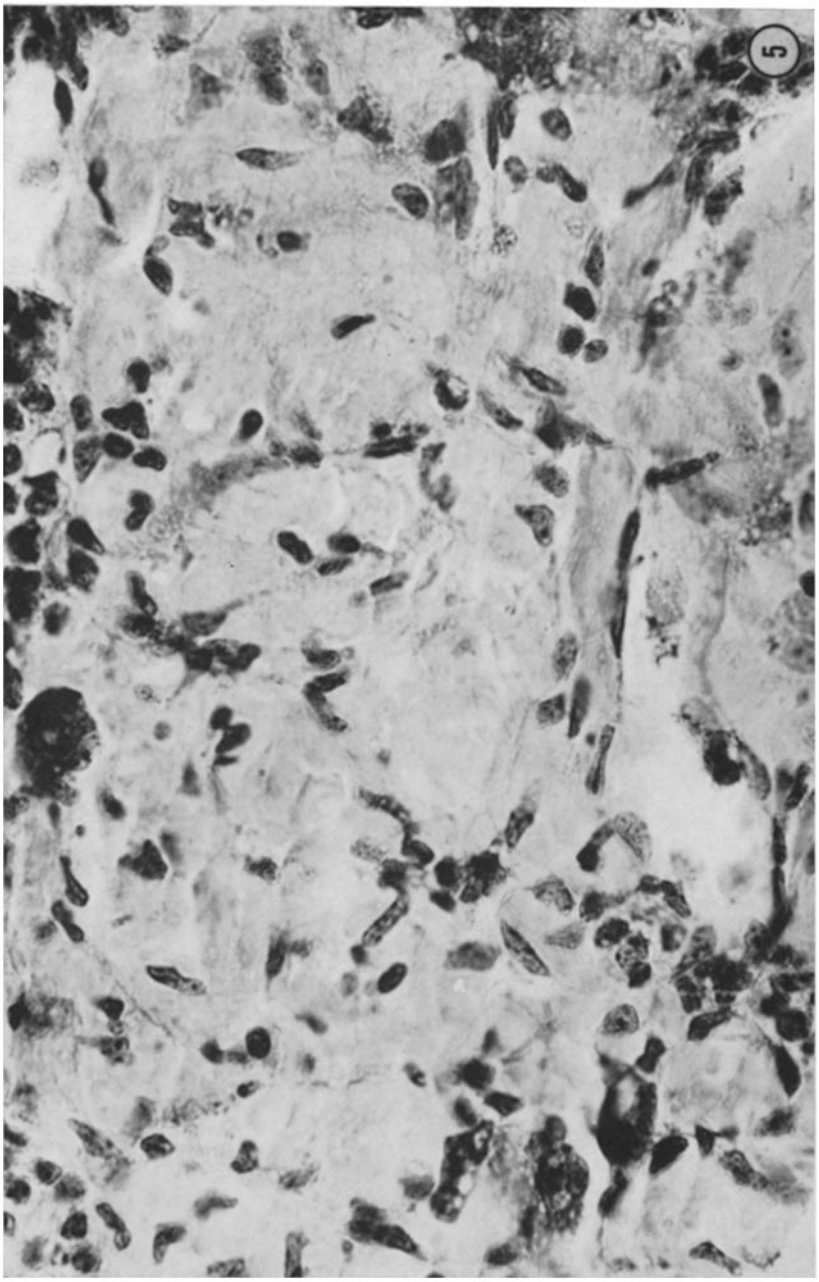




(Ebbesen: Spontaneous amyloidosis and thymus fibrosis)

PLATE 53

FIG. 5. Dying PAS cells in amyloid ovary from 15-month-old untreated CBA female mouse. Remnants of PAS cells are seen as numerous clusters of small PAS-positive granules in the amyloid. PAS technique. × 840.

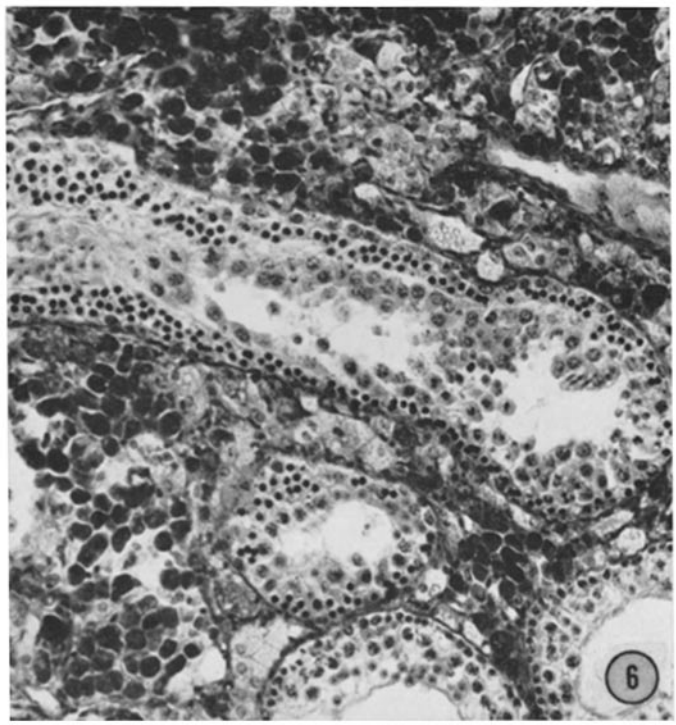


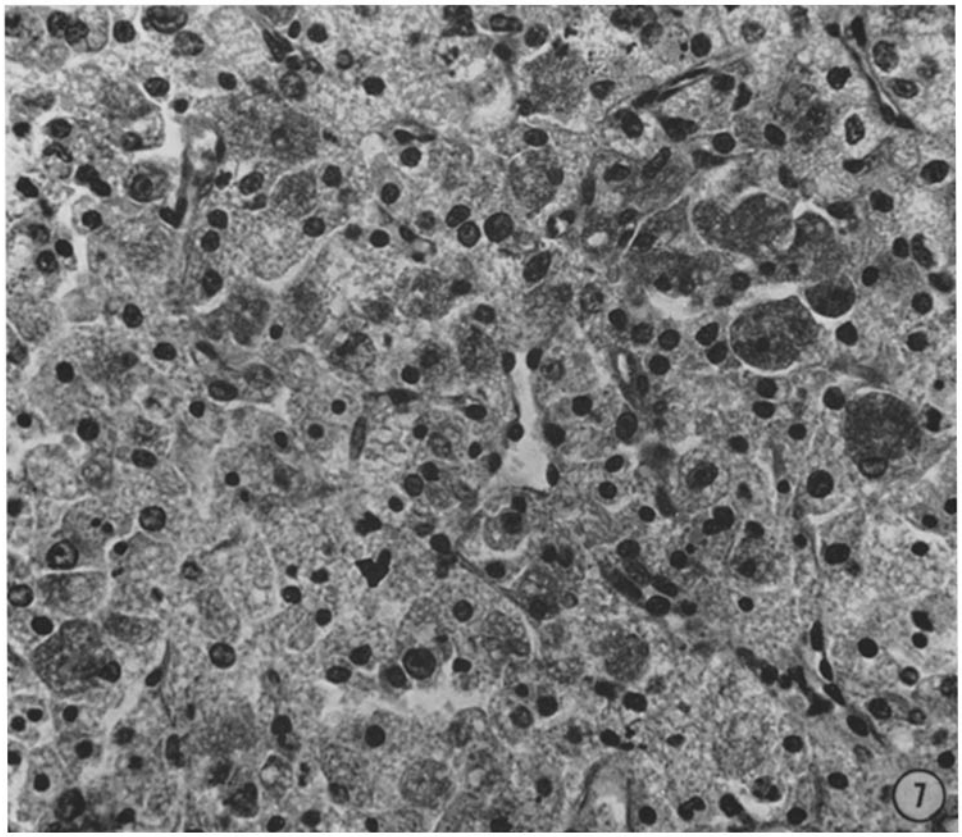
(Ebbesen: Spontaneous amyloidosis and thymus fibrosis)

PLATE 54

FIG. 6. Testes of BALB/c male treated with testosterone for 6 months. Note the numerous deeply PAS-positive interstitial cells. PAS technique. × 140.

FIG. 7. Section of interstitial cell tumor in BALB/c male treated with estrogen for 12 months. Note variation in staining intensity of cytoplasm in tumor cells. PAS technique. × 525.



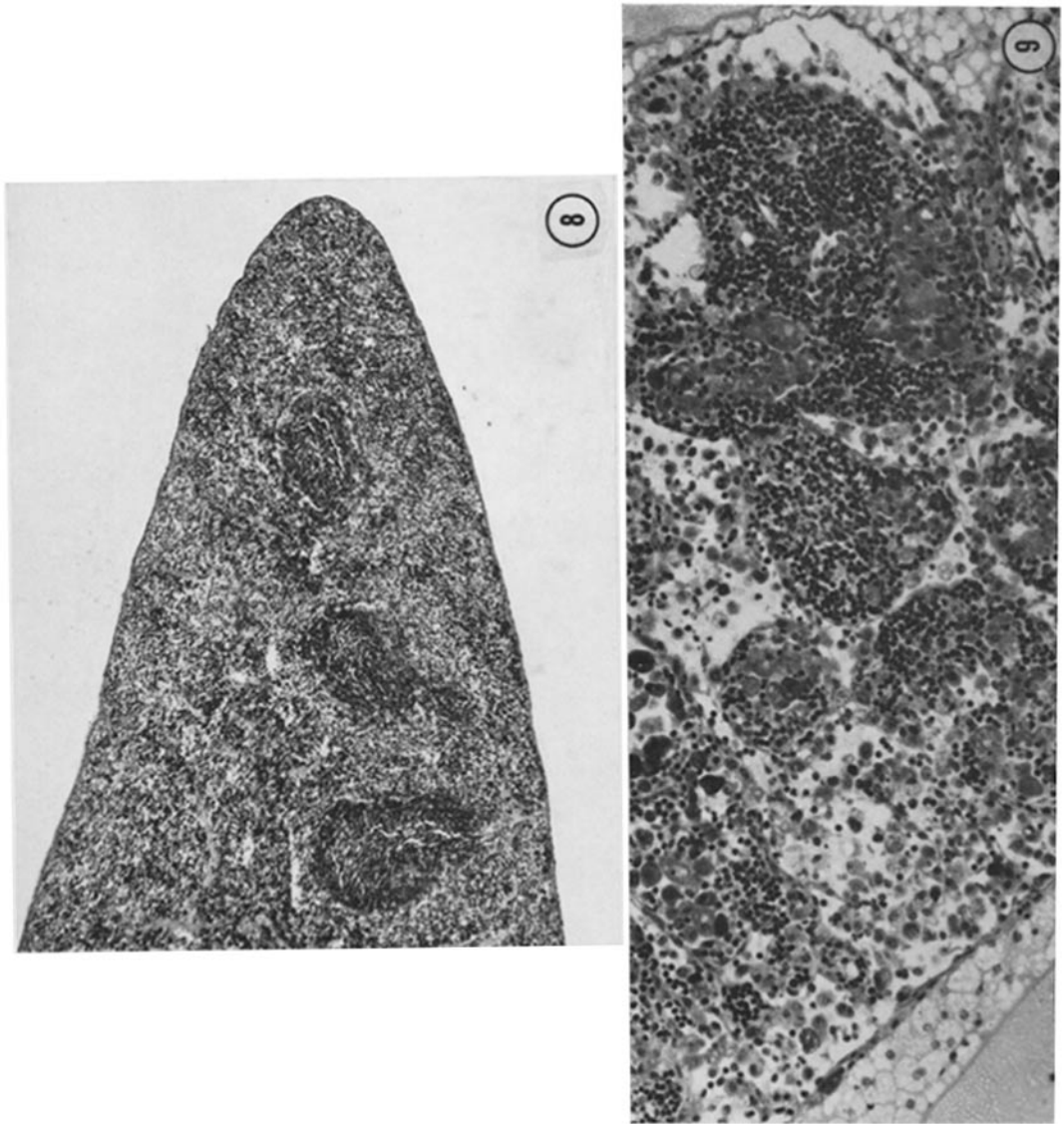


(Ebbesen: Spontaneous amyloidosis and thymus fibrosis)

PLATE 55

FIG. 8. Section of spleen from the same mouse as in Fig. 7. There is an extreme depletion of lymphocytes perifollicularly and no PAS cells or amyloid. PAS technique. × 52.

FIG. 9. Peripheral lymph node of same mouse as in Fig. 7. There is an extreme depletion of lymphocytes in the sinus; and many cells with ample cytoplasm of varying degree of PAS positivity. PAS technique. × 210.

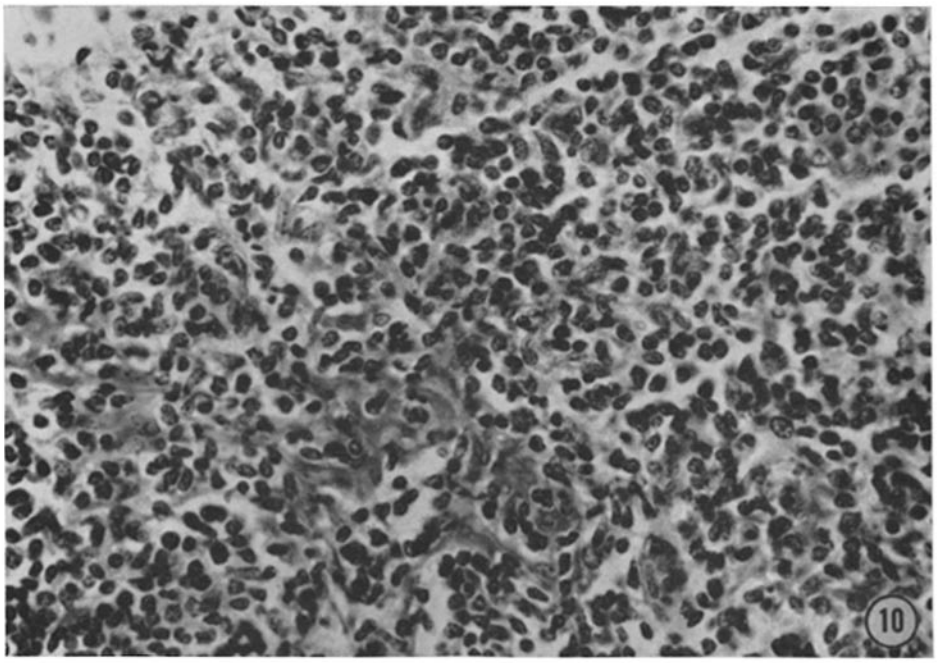


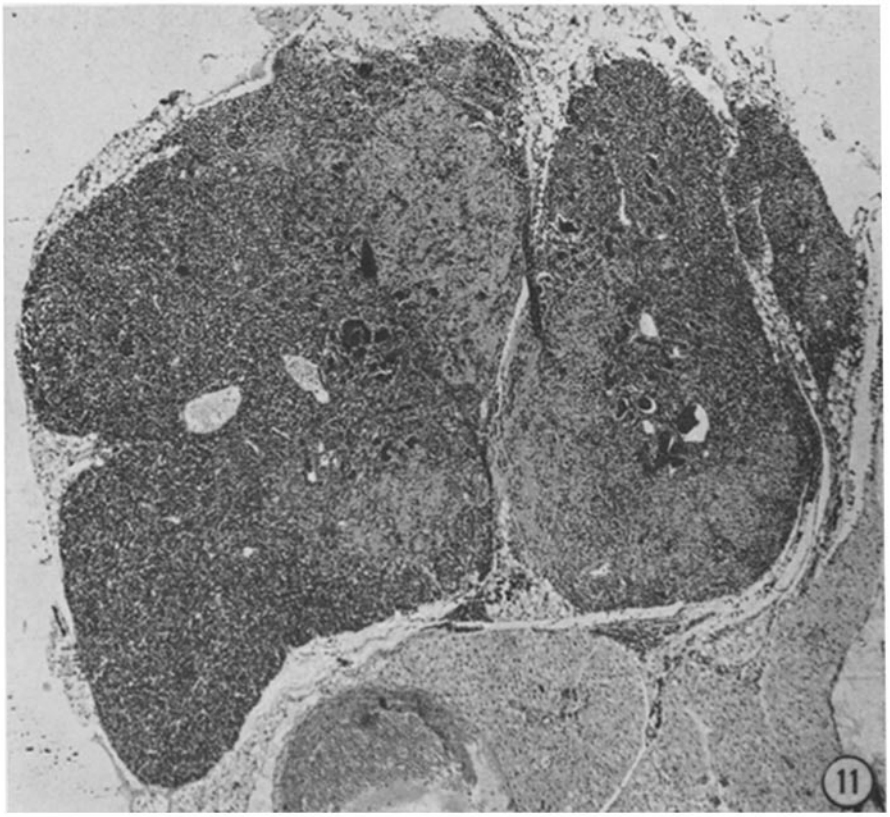
(Ebbesen: Spontaneous amyloidosis and thymus fibrosis)

PLATE 56

FIG. 10. Peripheral lymph node of 13-month-old estrogen-treated BALB/c male with testis tumor. Note small strands of fibrosis and depletion of lymphocytes. PAS technique. × 210.

FIG. 11. Thymus of the estrogen-treated BALB/c male bearing the interstitial cell tumor demonstrated in Fig. 10. Depletion of lymphocytes and PAS cells, interstitial fibrosis and big cysts with PAS-positive fluid prominent are seen. PAS technique. × 35.



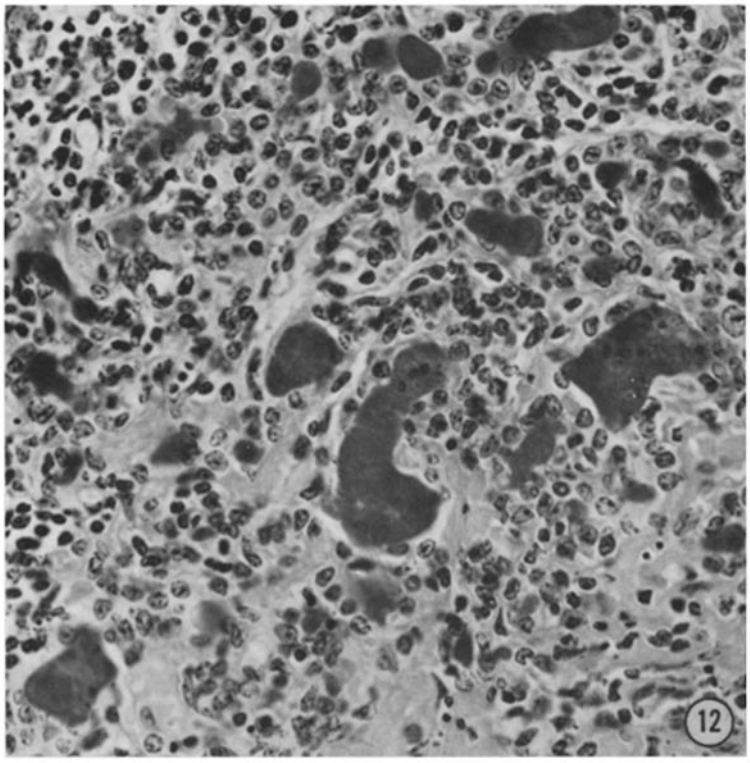


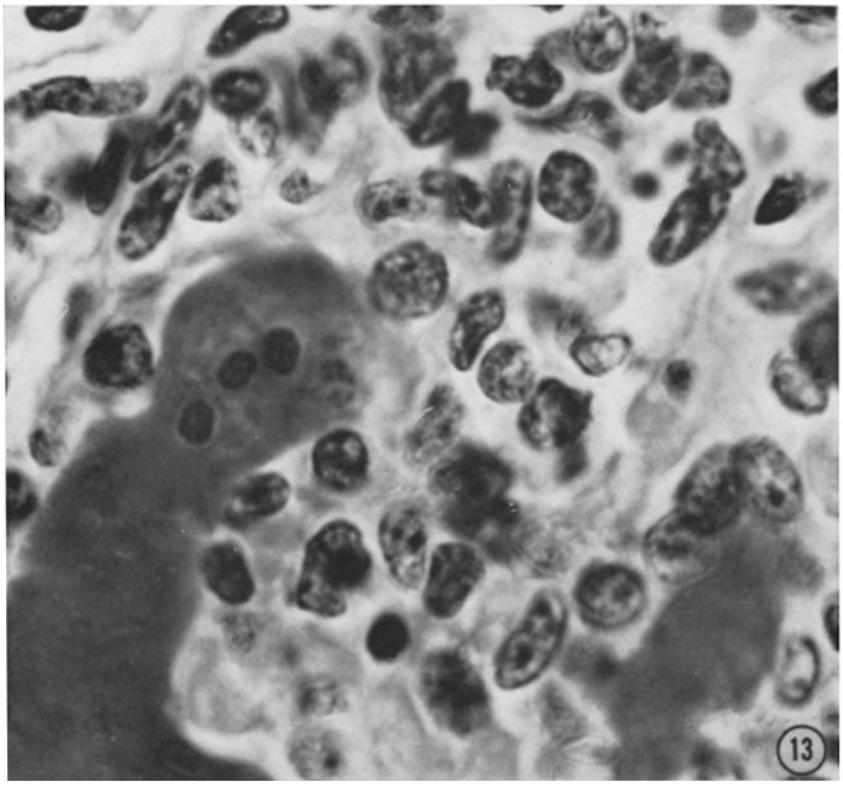
(Ebbesen: Spontaneous amyloidosis and thymus fibrosis)

PLATE 57

FIG. 12. Greater magnification of same thymus as in Figs. 10 and 11. Fibrotic strands which stained weakly with PAS and strongly red with van Gieson–Hansen, and big cysts in which the fluid contains many basophilic clumps are seen. Note necrosis of many lymphocytes in the surrounding area. PAS technique. × 500.

FIG. 13. Thymus cyst from the same mouse as Figs. 10–12. Necrotic nuclei are in the cyst fluid. Epithelioid and fibroblast-like cells are seen around the cysts. PAS technique. × 2000.





(Ebbesen: Spontaneous amyloidosis and thymus fibrosis)