

Melanotic Lesions of the Eye in August Hooded Rats Induced by Urethan or *N*-Hydroxyurethan Given During the Neonatal Period: A Histopathological Study^{1,2}

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SUMMARY—Beginning on the first day of life, inbred rats of the August hooded strain were given, at weekly intervals, a total of 8 subcutaneous injections of 1 mg/g body weight urethan (U) or *N*-hydroxyurethan (NHU). After 16 weeks, several rats developed macroscopically visible melanotic lesions of the eyes. All 15 U-treated males and 11 of 17 U-treated females that survived more than 16 weeks had melanotic lesions of one eye or both eyes. Of the NHU-treated rats surviving more than 16 weeks, 11 of 21 males and 11 of 21 females had melanotic lesions of one eye or both eyes. Histopathologically, the lesions were of two kinds, melanosis and melanoma. In different animals, they affected the iris, ciliary body, and/or choroid. None of 8 melanomas of the iris was invasive, but 2 of 3 melanomas of the choroid showed extension through the sclera. No distant metastases were seen.—*J Nat Cancer Inst* 43: 749–762, 1969.

INDUCTION of melanotic lesions of the eye in rats by urethan given during the neonatal period was first described in a preliminary report by Roe, Millican, and Mallett (1). In 40% of hooded rats of the August strain, urethan (8 subcutaneous injections of 1 mg urethan/g body weight given at weekly intervals starting on the first day of life) induced melanotic tumors of the iris. No lesions of the eye were seen in 250 untreated control August rats or in Wistar rats, whether or not treated with urethan in the same way.

The purpose of the present experiment was to see whether the earlier observation could be confirmed and, if so, to make a more detailed histopathological study of the lesions induced in the eye. At the time the experiment was started, there was speculation that urethan might only be active as a carcinogen if

converted to *N*-hydroxyurethan. At the suggestion of Professor E. Boyland of the Chester Beatty Research Institute, therefore, we included in the experiment a group of rats treated with the proposed *N*-hydroxylated metabolite of urethan.

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MATERIALS AND METHODS

Inbred August hooded rats from a subline maintained in the Chester Beatty Research Institute for several years by brother \times sister mating were used. One hundred and sixty-five newborn rats, obtained from 33 litters born over a period of 10 weeks, were allocated randomly to 6 groups. Urethan (U), obtained from Hopkin and Williams Lt., Chadwell Heath, Essex, England, and *N*-hydroxyurethan (NHU), prepared by Dr. R. Nery of the Chester Beatty Research Institute, were dissolved in sterile distilled water. During the first 24 hours of life, and thereafter once weekly for 8 weeks, the rats were given subcutaneous injections in the interscapular region of 10% w/v solutions of U or NHU. On each occasion, the dose was 1 mg/g body weight. The numbers of rats in the various treatment groups are shown in table 1. Metal cages were used for breeding; later the sucklings were housed in metal cages with their mothers until they were 4 weeks old, after which they were weaned, numbered on the ears, and rehoused, also in metal cages, according to group and sex, 8–11 per cage. Rats were fed a cubed diet (Formula 41B—supplied by Messrs. Dixon, Ware, Herts, England) and given tap water *ad libitum*. They were inspected every day and examined thoroughly once a week. Sick rats were killed and carefully examined post mortem. A few in each group that died during the night were eaten by others. Survivors were killed between 57 and 68 weeks after birth, and a full postmortem examination was carried out on each. The eyes

and other organs showing any abnormalities were removed and fixed in Bouin's solution. Paraffin sections, prepared at 5 μ , were stained with hematoxylin and eosin.

RESULTS

Eyes and Eye Changes

The first melanotic lesions of the eye, in the form of "dark spots," were observed at the end of the 16th week in 1 male and 2 females of the U-treated group and in 1 female of the NHU-treated group. During 15½ months of observation, 5 male and 5 female U-treated rats developed macroscopic melanotic lesions of the eye (*see* table 1). The corresponding figures for NHU-treated rats were 1 macroscopic lesion in the males and 2 in the females. Both eyes of the same animal were involved in 1 U-treated male and in 2 U-treated females. No macroscopic melanotic lesions were seen either during the experiment or at its termination among the 21 male and 25 female controls. The relationship between macroscopic appearance of eyes during life and histopathological changes is shown in table 2, and details of melanotic eye lesions discovered by microscopic examination are given in table 3.

Eyes in Control Rats

The first task in the histopathological examination of the eyes was to study in detail the normal histology of the eye of the August hooded rat.

TABLE 1.—Incidence of melanotic lesions in eyes of treated and untreated August hooded rats*

Treatment	Sex and No. of rats at start of experiment	Number of rats alive at time (16 wk) first lesion was seen	Number of rats that died after 16 weeks		Number of rats that died between 16 and 68 weeks with 1 or more melanotic eye lesions
			Without histopathological examination of eyes*	With histopathological examination of eyes	
Urethan	♂, 21	19	4	15	15
	♀, 28	26	9	17	11
<i>N</i> -Hydroxyurethan	♂, 28	27	6	21	11
	♀, 30	28	7	21	11
Control, untreated	♂, 30	27	6	21	0
	♀, 28	27	2	25	0

*Table refers to all eye lesions, whether seen during life or discovered only by microscopic examination.

TABLE 2.—Relationship between macroscopic appearances of eyes during life and histopathological changes*

Rat No.	Treatment group and sex†	Eye	Time of appearance of lesion (wk)	Duration of lesion before death (wk)	Histopathological lesions present				
					Melanosis of iris	Melanoma of iris	Melanosis of ciliary body	Melanosis of choroid	Melanoma of choroid
1	U, ♂	L+	21	47		+	+	+	
2	U, ♂	L							
3	U, ♂	R+	23	40		+	+		+
4	U, ♂	L+	16	47	+			+	
5	U, ♂	R	23	38					
6	U, ♀	L+	17	40	+		+		
7	U, ♀	R+	37	20		+	+		
8	U, ♀	L+	31	32	+				
9	U, ♀	R+	24	39		+	+		
10	U, ♀	L+	16	47			+		
11	U, ♀	R	58	5	+				
12	U, ♀	L							
13	U, ♀	R+	16	47		+		+	
14	NHU, ♂	L+	32	26					
15	NHU, ♂	R							
16	NHU, ♂	L	36	22		+			
17	NHU, ♂	R+	56	7				+	
18	NHU, ♀	L							
19	NHU, ♀	R+	16	47	+		+	+	
20	NHU, ♀	L+	24	39	+		+	+	
21	NHU, ♀	R							

*Table refers only to lesions observed during life (cf. tables 1 and 3).

†U = urethan; NHU = *N*-hydroxyurethan.

TABLE 3.—Melanotic lesions of the eye discovered by microscopic examination*

Treatment group†	Sex and No. of rats with lesions	Histopathological lesions present				
		Melanosis of iris	Melanoma of iris	Melanosis of ciliary body	Melanosis of choroid	Melanoma of choroid
U	♂, 10	10	0	1	4	0
	♀, 6	4	0	0	2	0
NHU	♂, 10	9	0	0	1	2
	♀, 9	8	0	0	2	0

*Table refers only to lesions discovered at death (cf. tables 1 and 2).

†U = urethan; NHU = *N*-hydroxyurethan.

Our observations on the eyes of the control rats are summarized as follows:

The iris, in horizontal sections, is very thin (fig. 1). The anterior surface is covered by a delicate layer of endothelial cells. Immediately under these cells is a loose connective-tissue stroma. Within this layer are blood vessels, a few melanocytes, and the sphincter muscle of the pupil. The blood vessels form the bulk of the iris and generally run radially.

The muscle fibers of the pupillary sphincter are well circumscribed at the pupillary border of the iris. The pigment epithelium of the iris forms a continuous layer extending from the ciliary body over the posterior surface of the iris. The cytoplasm of the round or oval epithelial cells usually contains a small amount of brownish-yellow pigment.

Histologically, *the ciliary body* is characterized by 2 or more ciliary processes. These are covered

TABLE 4.—Incidence of melanotic lesions of the eye induced by urethan (U) or *N*-hydroxyurethan (NHU) in August hooded rats*

Histological type and site of lesion	U-treated rats examined histopathologically		NHU-treated rats examined histopathologically	
	15 ♂	17 ♀	21 ♂	21 ♀
Melanosis of iris	12	6	9	10
Melanoma of iris	4	4	0	0
Melanosis of ciliary body	5	2	0	2
Melanosis of choroid	6	3	2	4
Melanoma of choroid	1	0	2	0

*Like table 1, this table refers to all eye lesions whether visible during life or not.

usually by 2 rows of oval epithelial cells. Melanin pigment granules are only in cells of the deeper layer. The stroma of the ciliary processes contains capillaries and a few melanocytes and is separated from the epithelial layer by Bruch's membrane (fig. 2).

Histologically, the choroid consists mainly of the capillary layer. The suprachoroid layer and the lamina vitrea cannot be distinguished in the eyes of August rats. The capillary layer contains thin-walled vessels embedded in choroidal stroma. This stroma is built up by connective tissue fibers, among which are a few pigmented cells of various sizes and shapes.

In the retina, all layers found in the normal human eye can be recognized. The only difference is the paucity of pigment cells (fig. 3).

Eyes in Treated Rats

Melanotic lesions were seen microscopically in U-treated and NHU-treated rats of both sexes. Not confined to the iris, the lesions were observed also in the ciliary body and choroid. Single and multiple lesions occurred and could be classified as *a*) melanosis and *b*) melanoma. The numbers of rats with lesions of the different types are shown in tables 2 and 3, and the total numbers of lesions induced by the two treatments in table 4.

a) *Melanosis*.—This, the most frequent lesion, was characterized by the presence of many deep-brown pigment-containing cells in the endothelial cell layer and stroma of the iris (fig. 4), in many parts of the ciliary body (fig. 5), and in some segments of the choroid. Some lesions caused marked thickening of

the affected structures, but there never was evidence of necrosis or loss of structure.

b) *Melanoma*.—This type of melanotic lesion led either to a peculiar deformation of the iris, usually at the pupillary margin (fig. 6), or to a diffuse thickening of some parts of the choroid. Microscopically, the structure of the iris was completely destroyed, so that its layers could not be recognized. The tumor cells were round or oval, and their cytoplasm was filled with fine or rough, deep-brown pigment granules; these cells occupied the whole iris (fig. 7). Melanoma of the iris sometimes was accompanied by melanosis of the ciliary body and by the presence of melanin-containing cells in the stroma of the cornea (fig. 8). Melanomas of choroid were found in 2 NHU-treated males and 1 U-treated male. Both the NHU-induced melanomas presented as diffuse thickenings of the choroid in the neighborhood of the optic nerve (fig. 9), and showed extraocular extension (fig. 10). From their morphological similarities to the flat type of melanoma of choroid in man [see fig. 53, in Reese, 1956 (2)], they have been classified by us as melanomas. Microscopically, the only melanoma seen in a U-treated male rat consisted mainly of spindle-shaped and epithelial-like cells with a low content of pigment and showed occasional mitotic figures (fig. 11) but no evidence of extraocular extension.

Neoplasms of Other Sites

Only 3 neoplasms of other sites were found: a mammary adenocarcinoma in a U-treated female, a mammary fibroadenoma in an NHU-treated

female, and an islet-cell adenoma of the pancreas, also in an NHU-treated female.

DISCUSSION

U or NHU injected subcutaneously into newborn August hooded rats induced a variety of melanotic lesions in the eye, not previously shown to be susceptible to the induction of neoplasms by chemical carcinogens. The results reported here together with our previous findings (1), suggest that the uveal melanocyte system of newborn August hooded rats is particularly susceptible to the parenteral administration of the compounds in question.

The neoplastic effects of U have been studied in various strains of newborn and adult mice, hamsters, and rats (3-8). NHU has been studied less extensively (9), but no one, except us, has reported inducing lesions of the eyes by either U or NHU. NHU proved more or less as effective as U in inducing melanotic eye lesions under the experimental conditions used. Only 3 neoplasms of sites other than the eye were found in the present experiment. Moreover, all these tumors were of types frequent in untreated rats of the same strain, so that there are no good grounds for assuming that they were induced by U or NHU.

It is tempting to regard the two kinds of histological lesion—melanosis and melanoma—as two stages of a continuous process, but there is no proof that they are temporally related in this way. If there are two stages, melanosis of the iris, ciliary body, and choroid may be a first stage from which, in some cases, melanomas develop characterized by cellular pleomorphism, invasiveness, and destruction of normal tissues. Alternatively, the two types of lesion may differ *ab initio*.

The lesions induced by U and NHU in August rats closely resembled melanotic lesions of the iris and choroid in man. Therefore, it seemed sensible to use the same terminology as ophthalmic pathologists concerned with human material (2, 10). As in man, the melanomas of the iris and choroid in the present experiment markedly altered the shape and size of the affected parts of the eye and led to the total loss of their original structure.

It seems likely that most lesions originated from uveal melanocytes rather than from pigment

epithelium, since in most instances the pigment epithelium was apparently intact (e.g., fig. 4).

Most eye lesions induced by U or NHU were clearly benign. Lesions visible during the early stages of the experiment in 13 rats (table 2) tended to enlarge slowly over observation periods of between 5 and 57 weeks. Extraocular extension was seen only in 2 rats, and even in these, spread appeared to be limited to the adjacent tissues of the orbit. No distant metastasis was noted. Despite the nonaggressive nature of most lesions, it seemed appropriate to regard a proportion of them as melanomas. The criteria for this histological diagnosis were based on the cell pattern, the degree of anaplasia, the number and nature of mitoses, and the presence of invasion. The type of melanoma of the iris illustrated in figures 6 and 8 was regarded as malignant by Reese (2) in his assessment of human material.

Dogs, cats, horses, cattle, sheep, chickens, rabbits (11), and xiphophorus fish (12) develop spontaneous benign and malignant pigmented lesions of the eye. These arise from the iris, ciliary body, or choroid. To our knowledge, no neoplasms of the pigment epithelium have been reported in these species. However, Kurz and Zimmerman (13) believe true neoplasms may, albeit very rarely, arise from the pigment epithelium in man. They write: "Although true neoplasms arising from the retinal pigment epithelium are among the rarest of lesions, this layer proliferates under the slightest provocation. A variety of lesions, although obviously benign histologically, are capable of simulating malignant melanoma of the choroid clinically. In a few instances hyperplasia of the retinal pigment epithelium may take on characteristics of a true neoplasm." The etiology of the melanotic eye lesions in man and the animal species mentioned above is unknown. Spontaneous malignant melanotic tumors of the skin are rare in rats or mice, but do arise occasionally in Syrian golden hamsters (14). Nakai and Rappaport (15) report that the Syrian golden hamster is an excellent model for studying melanotic tumors of the skin which biologically and morphologically resemble the corresponding tumors in man.

After topical application, 7,12-dimethylbenz[a]-anthracene (DMBA) induced melanomas of the skin in Syrian golden hamsters (16-18). It also

induced melanotic tumors of the skin in white (partially albino) Syrian hamsters (19, 20). Another compound, 4-nitroquinoline-*N*-oxide, after prolonged painting on the dorsal skin, led to the development of melanomas in hamsters (21). Some melanotic tumors of the skin induced by DMBA in hamsters were transplantable (22, 23) and gave rise to melanotic and amelanotic tumor strains (24).

U is a multipotential carcinogen for Syrian golden hamsters (25), and, as reported by Pietra and Shubik (26) and later by Rivière and his colleagues (27), when given in the drinking water, it caused melanotic lesions on the skin of hamsters. Walters, Roe, and Levene (28) provoked melanotic tumors in the dermis of the dorsal skin and in the margins of the eyelids of hamsters by neonatal injection of DMBA, but not of U.

The histogenesis of the U-induced and NHU-induced melanotic lesions of the eye in August hooded rats is unknown. With regard to the histogenesis of uveal melanotic lesions of man, it is generally agreed that some lesions arise from the Schwann cells (2, 29). The hypothesis of the neurogenic origin of the carcinogen-induced melanotic tumors of the skin in hamsters was supported by an electron microscopic finding (30), namely, the presence of pigmented melanocytes within cutaneous nerves in hamsters painted with DMBA.

On the other hand, there is a growing belief that pigmented stromal cells of the uveal tract are typical melanoblasts actually producing the pigment deposited in their cytoplasm. Arey (31) stated: "In this way they are considered distinct from the chromatophores of the human skin which obtain their pigment by phagocytosis. . . . In formative stages, the choroidal elements are therefore true melanoblasts and can revive this potentiality when stimulated pathologically."

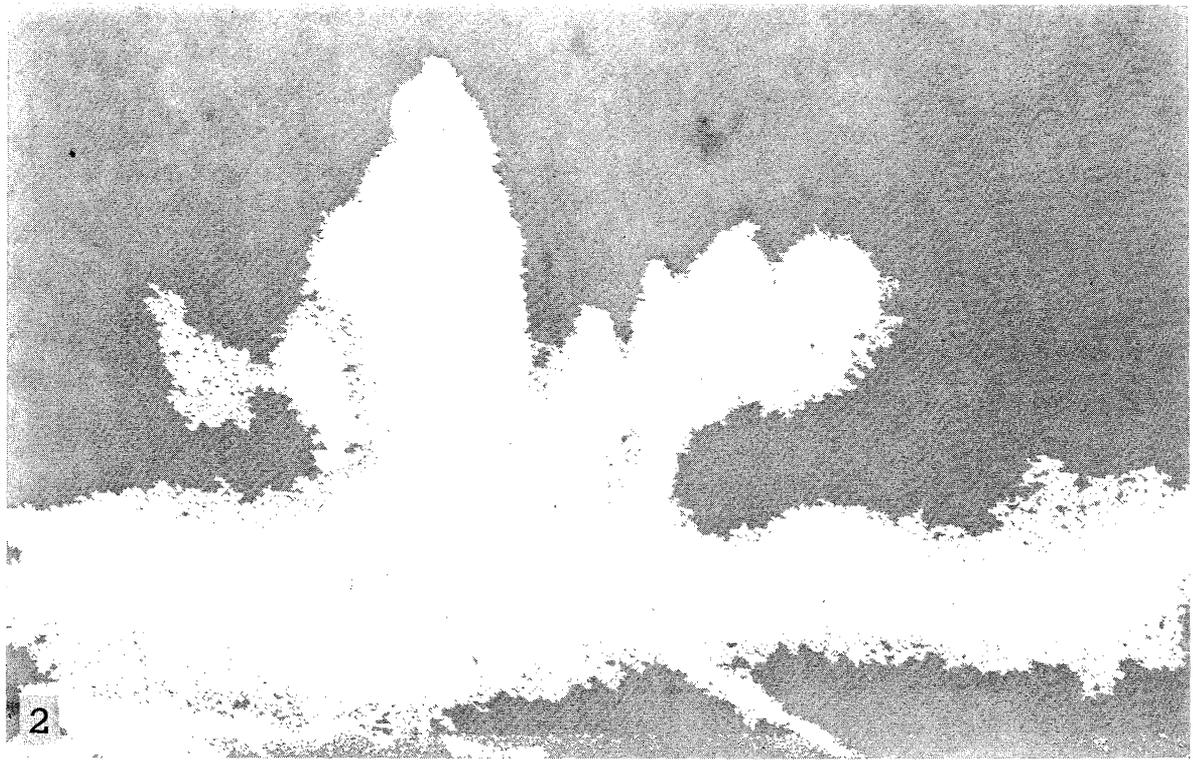
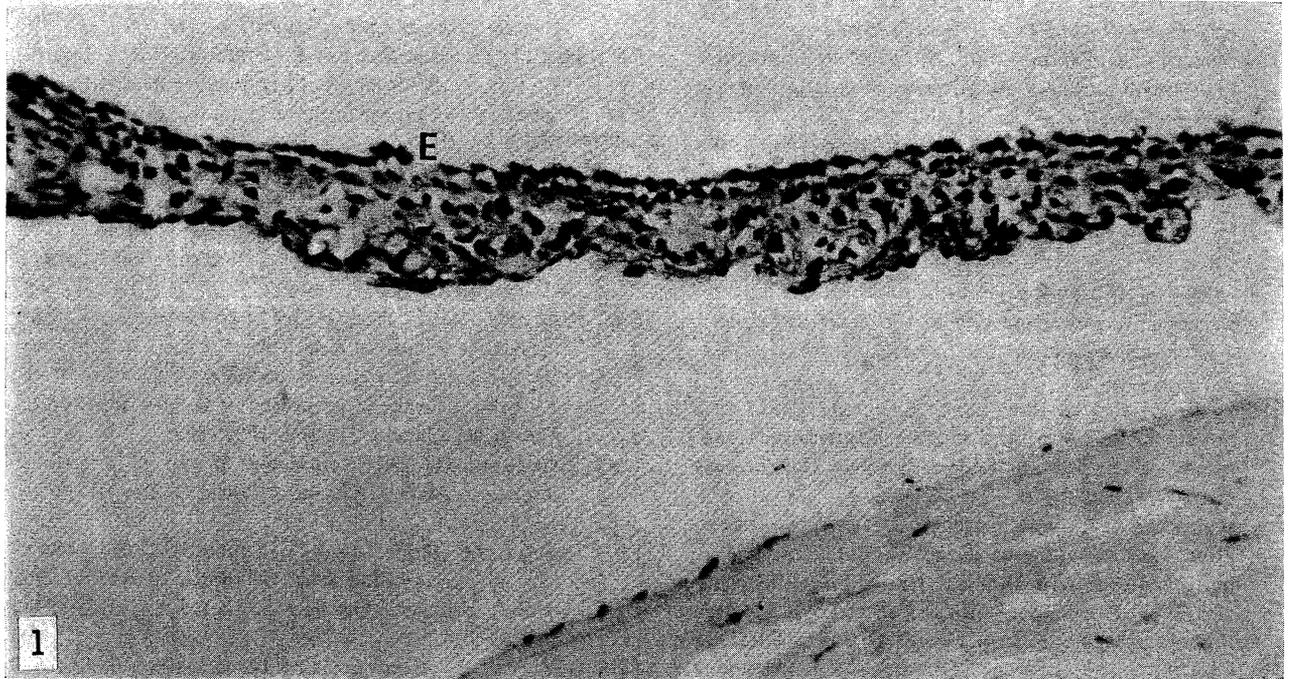
The present report suggests that administering U or NHU to very young August rats stimulates melanoblasts in the uveal tract. The time at which microscopically detectable changes first occur and the nature of the earliest changes are the subject of experiments now in progress.

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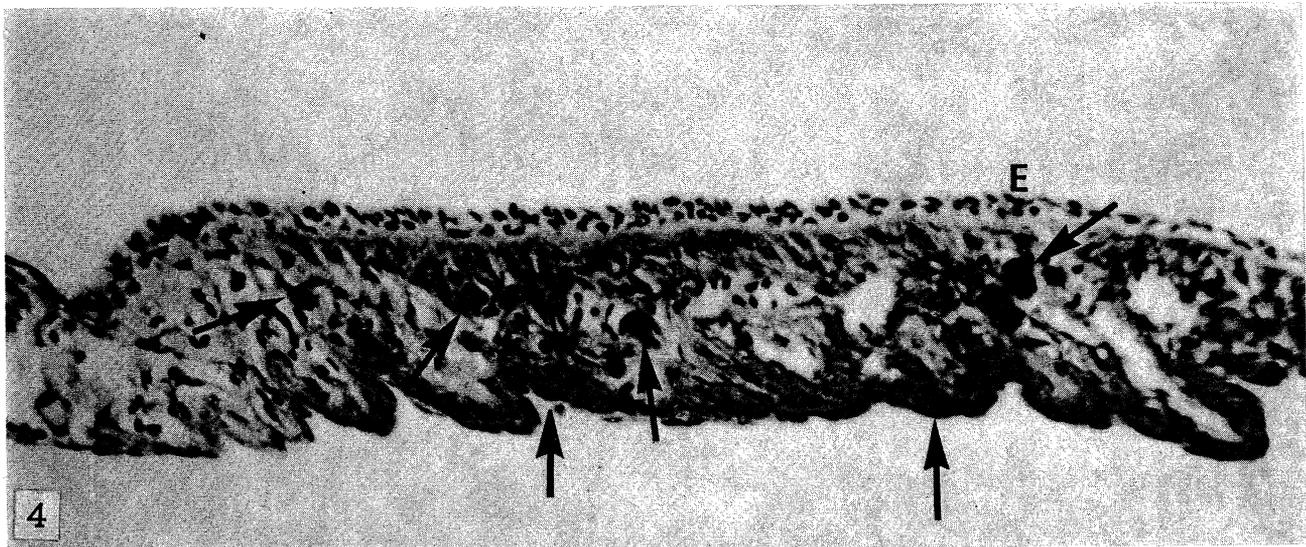
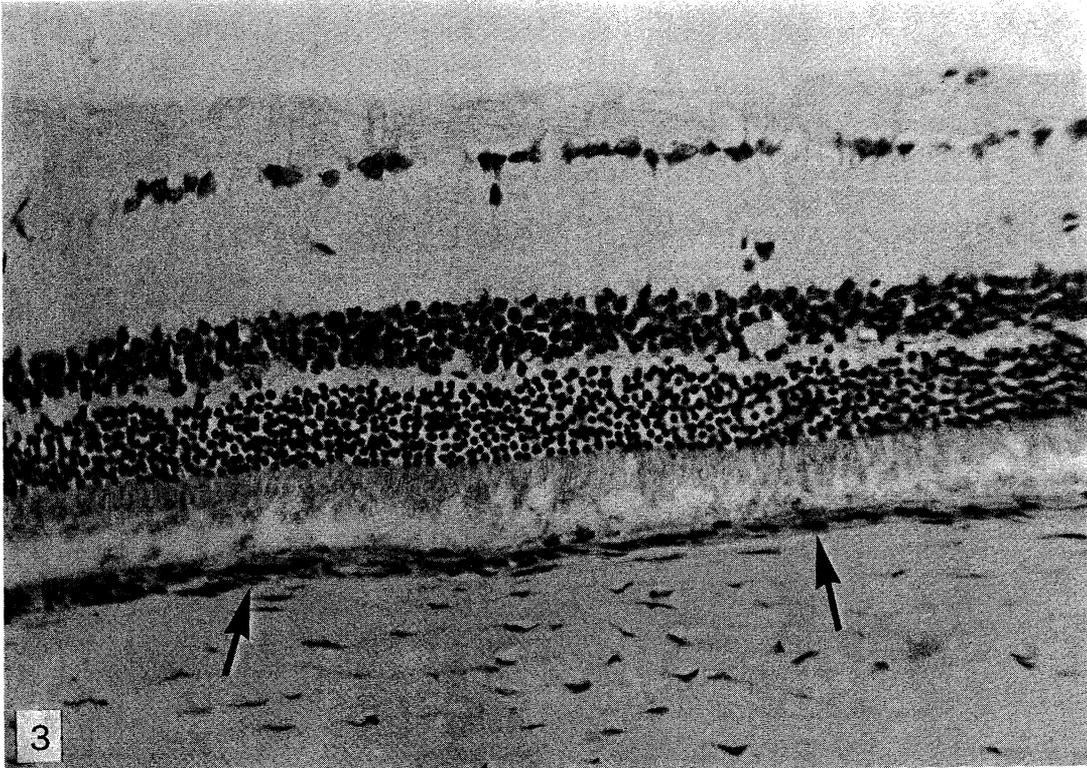


FIGURE 3.—Retina and choroid of male control rat. Thin choroida layer (*arrows*) contains a few pigment cells not well shown in this photomicrograph. There are no pigment-containing cells in retina. However, an expert commenting on this photomicrograph said: "Several cells beneath the photoreceptor layer resemble amelanotic retinal pigment epithelial cells." Hematoxylin and eosin. $\times 300$

FIGURE 4.—Melanosis of iris in a urethan-treated female August hooded rat. Iris is thickened conspicuously, and pigmented cells accumulate in stroma (*arrows*). Pigment epithelial layer (E) appears normal. Hematoxylin and eosin. $\times 300$

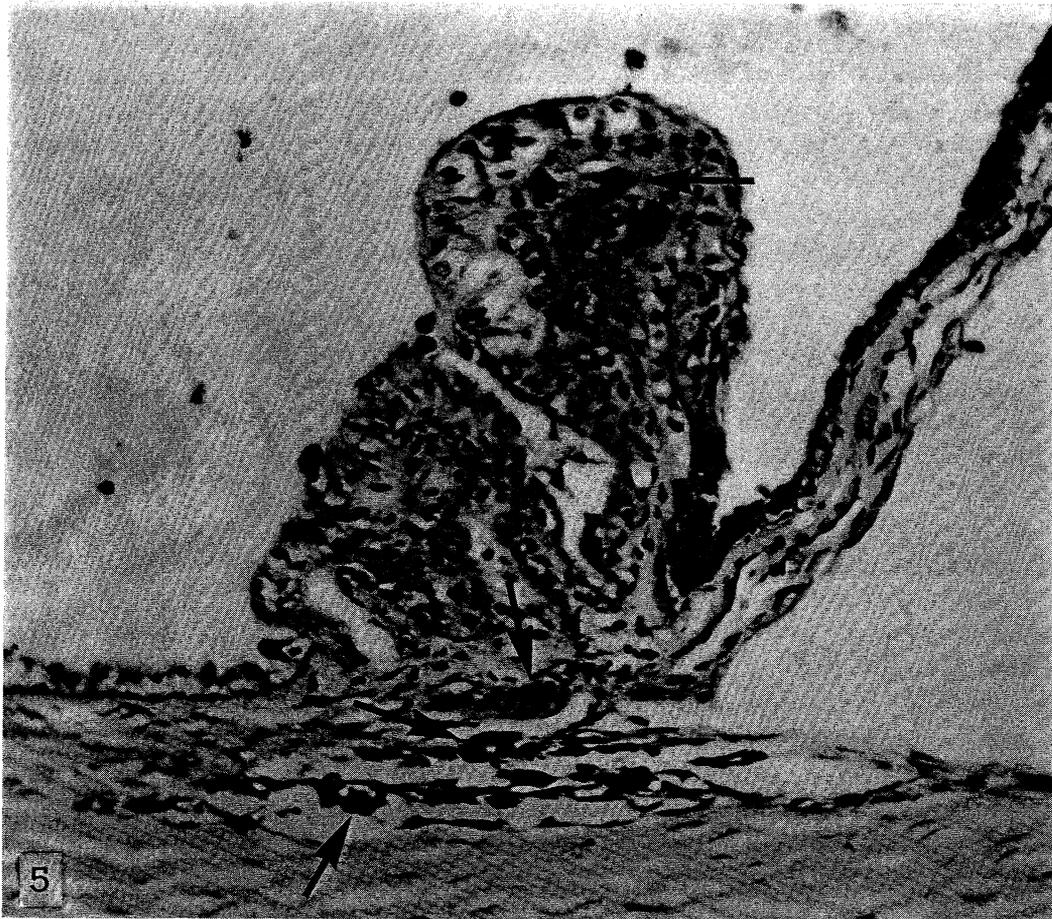
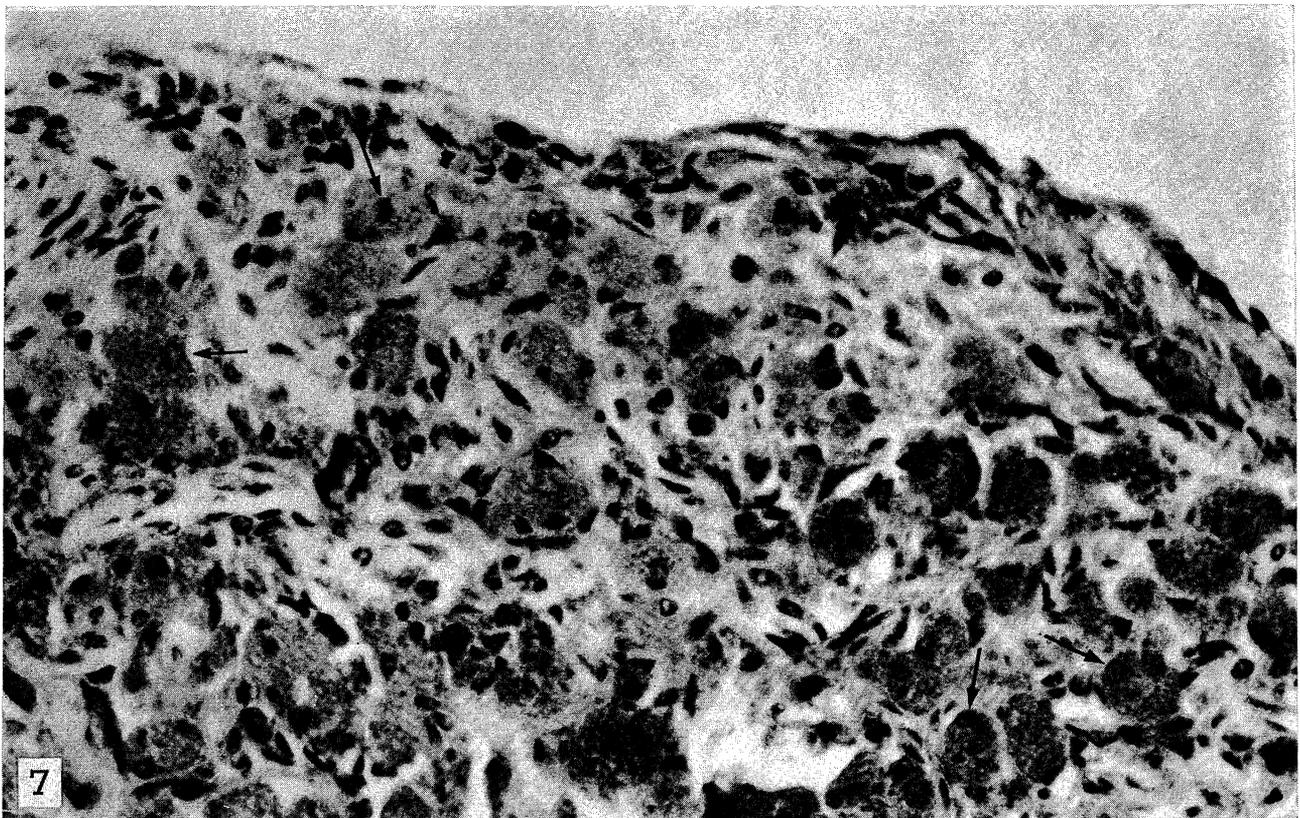
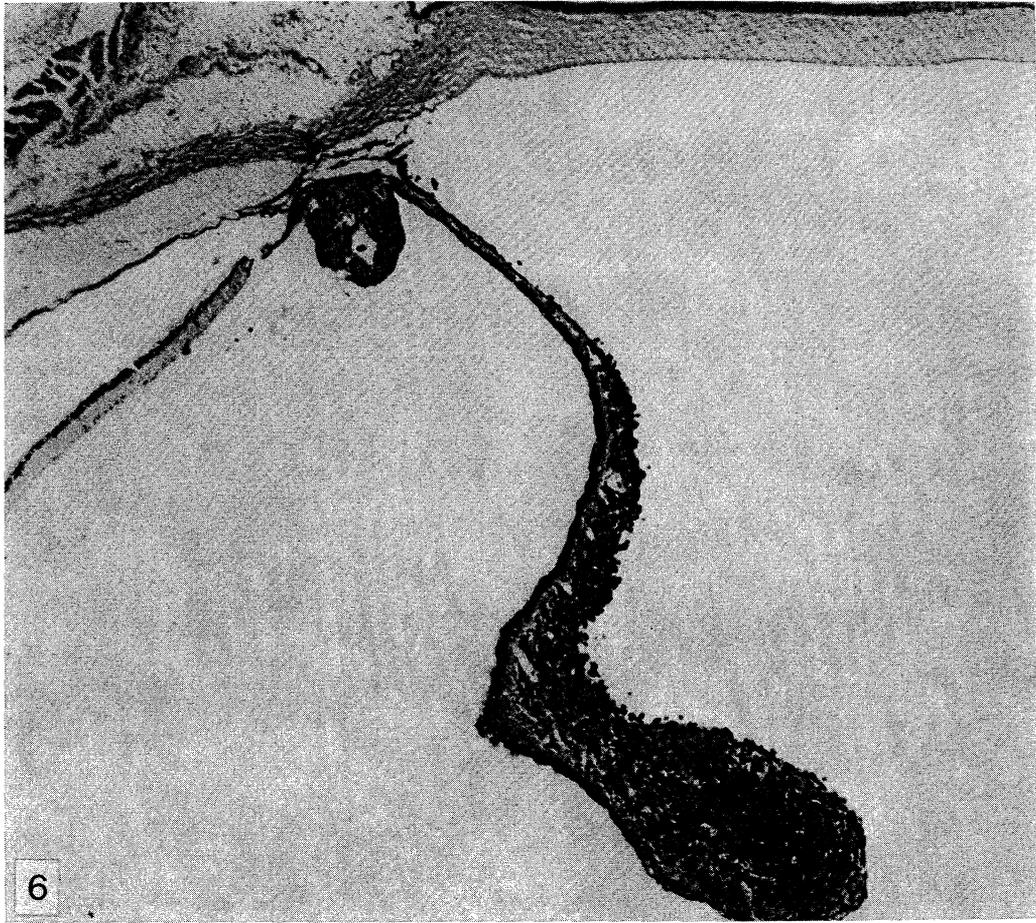


FIGURE 5.—Melanosis of ciliary body in a urethan-treated male August hooded rat. Number of melanocytes (*arrows*) has increased throughout stroma but particularly in its base. Hematoxylin and eosin. $\times 300$

FIGURE 6.—Melanoma of iris in a urethan-treated male August hooded rat. Iris is greatly thickened near papillary margin. The normal structure of the iris in this region is almost destroyed. Hematoxylin and eosin. $\times 300$

FIGURE 7.—Same tumor as that in figure 6. Lesion consists of pigmented cells of fairly regular shape and size interspersed with spindle-shaped connective tissue cells. Nuclei of pigment cells are small in relation to their size and pale. Only a few such nuclei (*arrows*) are easily recognized. Hematoxylin and eosin. $\times 300$



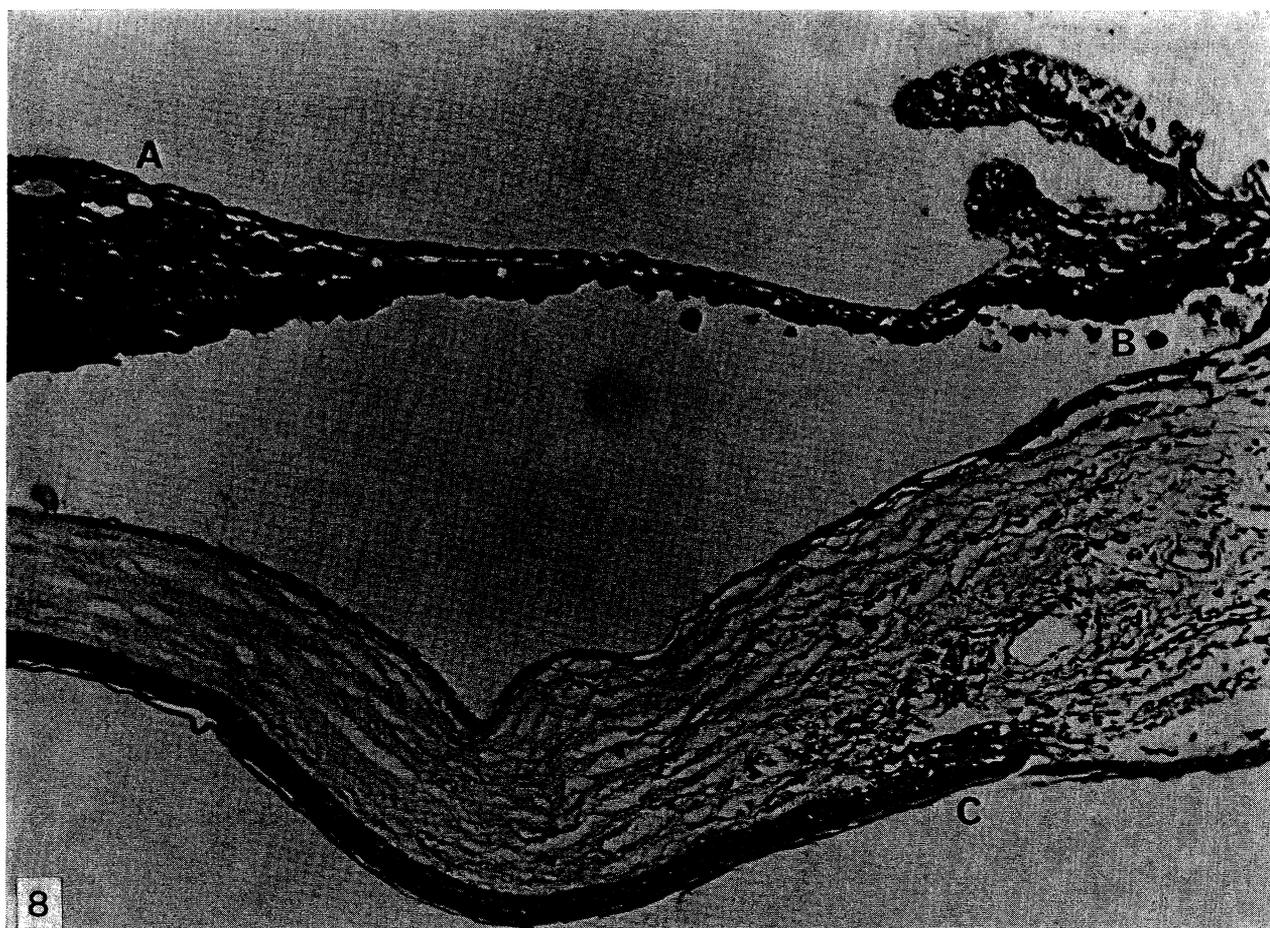
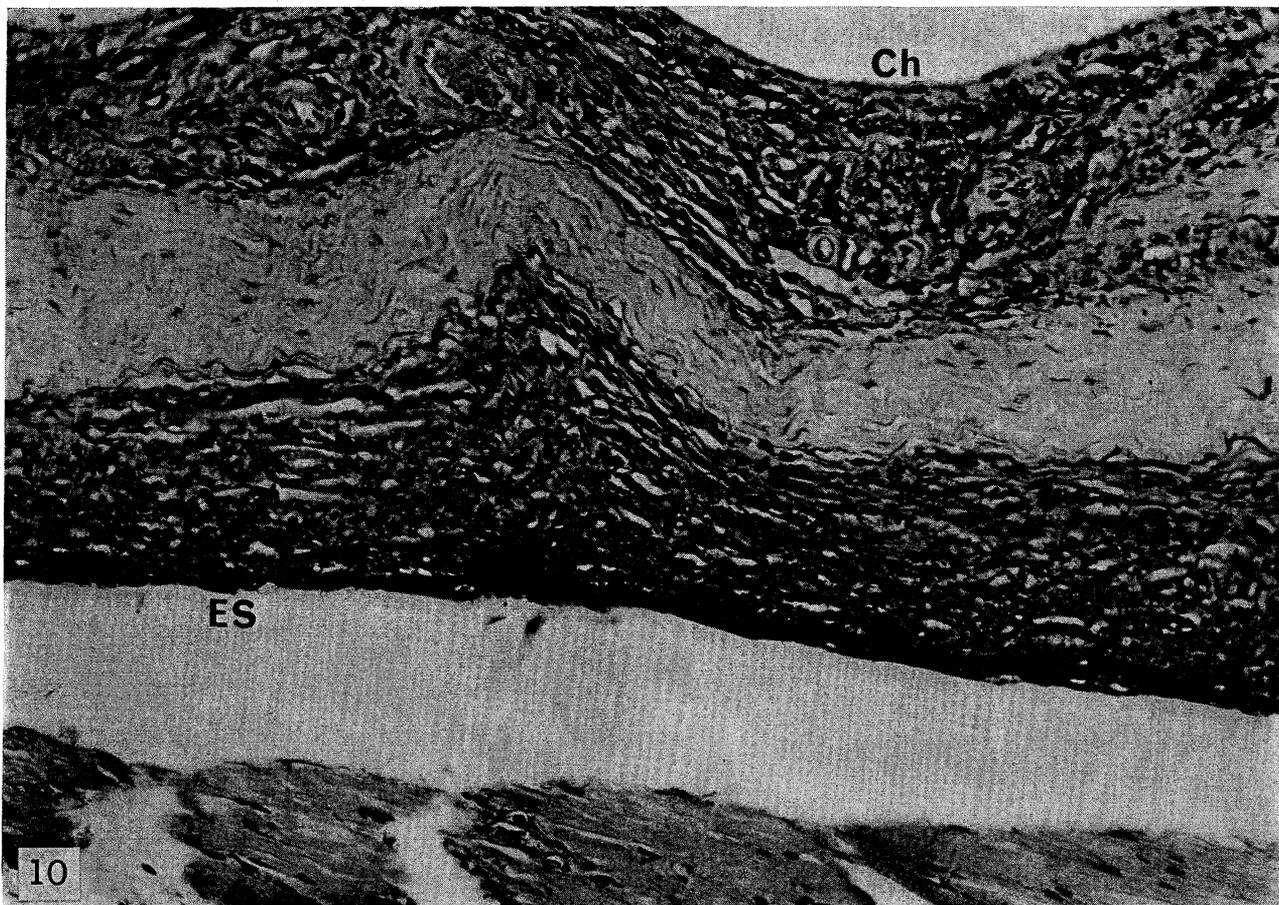
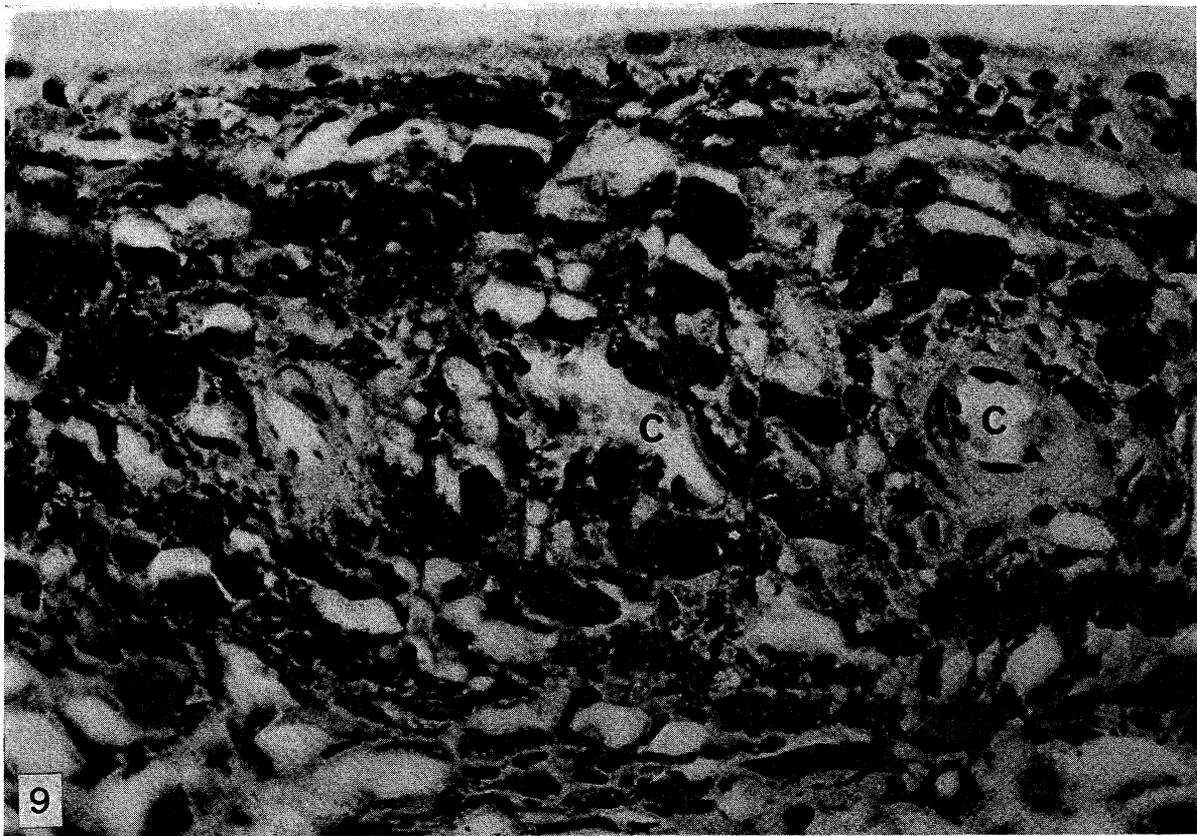


FIGURE 8.—Melanoma of iris (A), melanosis of the ciliary body (B), and pigment-containing cells in stroma of cornea (C) in a urethan-treated male August hooded rat. Hematoxylin and eosin. $\times 40$

FIGURE 9.—Melanotic lesion of choroid in an *N*-hydroxyurethan-treated male August hooded rat. *Note* remarkable thickening of choroid due to accumulation of large pigment-containing cells in stroma. Capillaries (C) and connective tissue cells can be identified. A similar lesion was found on the outside of the sclera. Hematoxylin and eosin. $\times 675$

FIGURE 10.—Melanotic lesion of choroid (Ch) in an *N*-hydroxyurethan-treated male August hooded rat, showing extrascleral extension (ES). Hematoxylin and eosin. $\times 160$



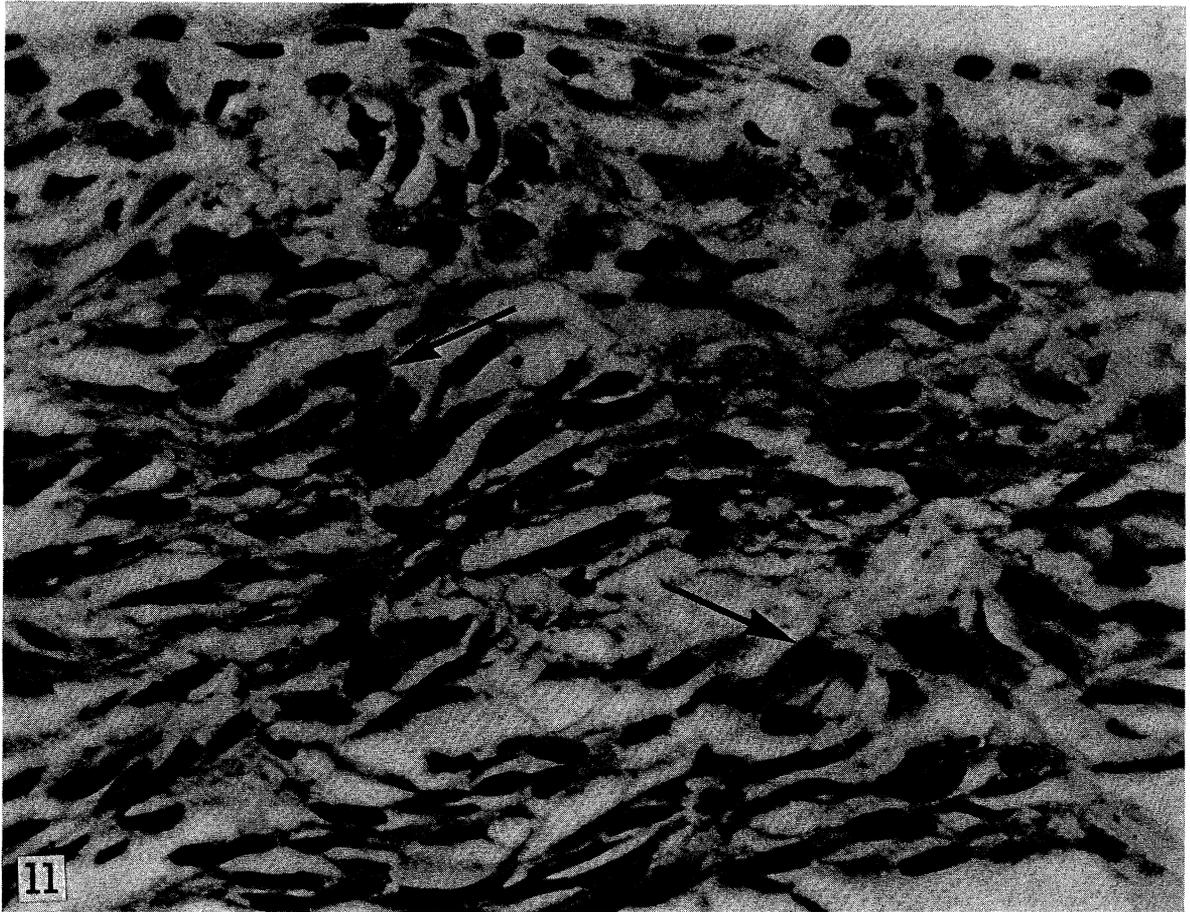


FIGURE 11.—Mainly amelanotic melanoma of choroid in a urethan-treated male August hooded rat. Lesion consists of pleomorphic cells, 2 of which are undergoing mitosis (*arrows*). Hematoxylin and eosin. $\times 880$