OCULAR MELANIN AND THE ADRENERGIC INNERVATION TO THE EYE*

BY *Alan M. Laties,* MD

INTRODUCTION

IT IS NOW SOME SIXTY YEARS SINCE CALHOUN PRESENTED HIS THESIS "CAUSES OF Heterochromia Irides with Special Reference to Paralysis of the Cervical Sympathetic"[1] to this Society. He sought to define the relationship between the sympathetic nervous system and iris heterochromia. To do so he documented iris heterochromia in patients he had seen who had Horner's syndrome and he duplicated his clinical observations in animal experiments — experiments to observe the effect that removal of the superior cervical ganglion had on iris color.

As a result of his work and of the work of Angelucci,[2] of Bistis,[3] and of several others, a relationship between the adrenergic innervation to the eye and iris heterochromia in Horner's syndrome was established. Collectively, their experimental work was as much as could be done at the time. It is a measure of the progress of biology that it is now possible to redo and to extend their labors to achieve a better understanding of the precise relationship between the adrenergic innervation and ocular melanin.

Several laboratory techniques are evidence of this progress. As a direct result of the development of specific neurohistological techniques for the demonstration of sympathetic nerves, it is now possible to visualize adrenergic nerves within tissues and in many instances to judge with reasonable accuracy their relationship to terminal structures, such as smooth muscle or melanocytes. In addition, biochemical assays which measure the activity of specific enzymes are now available. In the present context, reliance is placed on an assay of the rate-limiting enzyme in the biosynthesis of melanin. As a result, it is now possible to undertake experiments to define the role played by adrenergic nerves in the formation of melanin within the eye.

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The present thesis is based on such a set of experiments. The relationship of adrenergic nerve to melanocyte and the relationship of the enzyme tyrosinase to the synthesis of melanin are both examined. In order to encompass as many of the variables which bear on the development of melanin pigment as possible, selected results from a diverse set of experiments are presented. However, a common thread runs through all the experiments — they all have to do with the question: to what degree do the melanin-containing cells of the eye depend on the adrenergic innervation for the development of, or for the maintenance of their content of pigment?

In addition, just as in Calhoun's thesis, information gleaned from careful clinical examination of a patient with Horner's syndrome is presented, information which in this case bears a signal relationship to the laboratory studies which follow.

CASE PRESENTATION

A 29-year-old, white female was found on routine examination to have right-sided ptosis and miosis. When questioned as to its occurrence, she reported that when 14 years of age she had noted drooping of the lid immediately after surgery to the right side of her neck. Her hospital record was obtained and reviewed.

At the time of surgery a tissue block which measured 6 x 5 x 4 cm containing an encapsulated tumor had been resected from an area just deep to the carotid bifurcation. The tissue section revealed "dense eosinophilic tissue with many spindle-shaped nuclei in definite palisades". A diagnosis of neurolemmoma was made at the time. Excision had been total; there was no evidence of recurrence in 15 years.

In the current examination, visual acuity was found to be 6/6 in each eye when mild, bilateral, myopic astigmatism was properly corrected. As already noted, ptosis and miosis were apparent on the right. In addition there was an obvious difference in color between the two irides, the right iris being lighter than its fellow. On the left, the iris was brown with a slight greenish tint. On the right, the brown was washed out and the green predominated (Figure 1A and B). The patient was an artist and was acutely aware of color. She volunteered that it was puzzling to her but she believed the color of her right iris to be changing slowly. Ocular tensions were equal at 8/5.5 gram weight Schiøtz. Careful examination of both anterior segments revealed no other abnormalities. Specifically, there were no findings indicative of inflammation and neither iris transilluminated abnormally. Ophthalmoscopic examination was also within normal limits in each eye. In an attempt to ascertain if there were any differences in choroidal pigmentation, a series of fundus photographs were taken with a Zeiss fundus camera on Kodachrome film after maximal dilatation of the pupils. As shown in Figure 1C and D, there was a slight difference in choroidal pigmentation between the two sides,

although the difference was small. Just as was the case with the iris, the right side was lighter.

In an attempt to see if the small differences in color had a physiological implication, electroretinography and dark adaptation studies (Figure 2) were done. In each instance there was no significant difference between the two eyes.

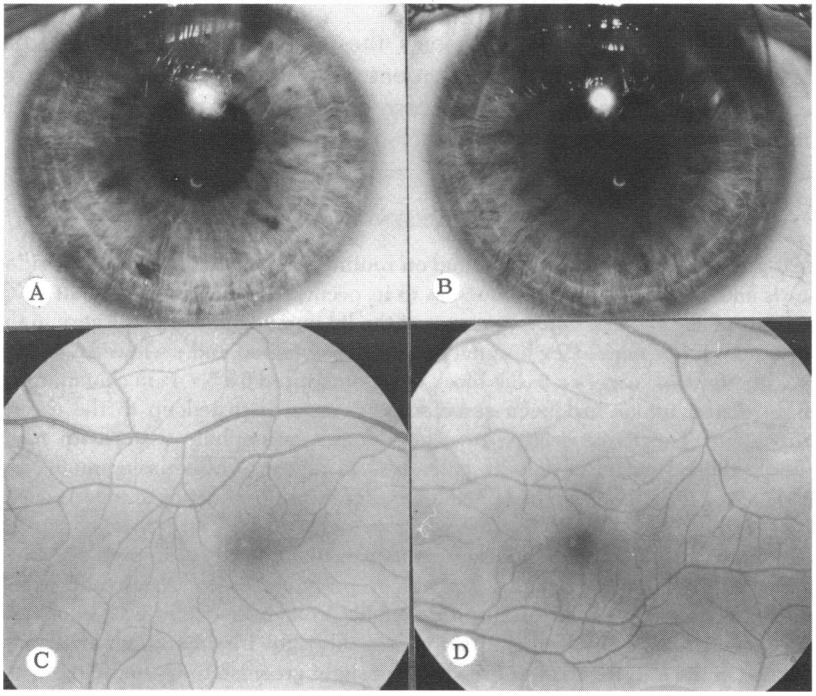


FIGURE 1

Horner's syndrome of 15 years duration in a 29 year old woman. Right (A) iris is hypochromic as well as miotic when compared to the left (B). The right ocular fundus (C) is less deeply pigmented than the left (D). Difference is minimal.

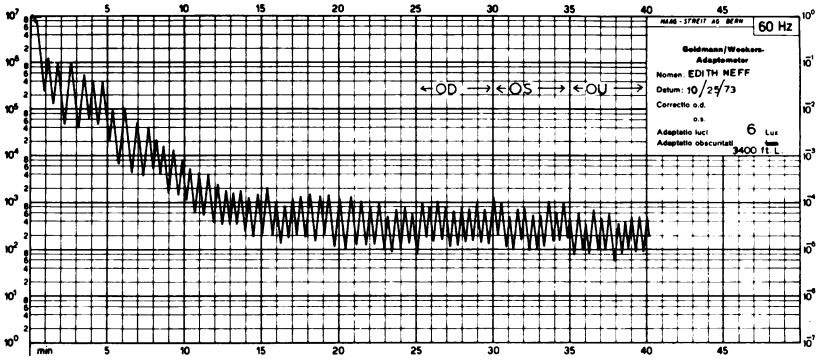


FIGURE 2
Horners syndrome of 15 years duration. There is no discernible difference in dark adaptation between the two eyes.

IRIS HETEROCHROMIA AFTER INTERRUPTION OF THE SYMPATHETIC INNERVATION TO THE EYE

A total of 28 Dutch-belted rabbits of either sex, average weight 2.3 kg, were used in this study. The animals were approximately six months of age. After induction of satisfactory barbiturate anesthesia or, alternatively, of halothane anesthesia, the superior cervical ganglion was removed in 10 animals, while in another 18 animals, the sympathetic trunk caudal to the superior cervical ganglion was resected for a length of 5 mm. In accordance with standard physiological terminology (see W.B. Cannon, 1937)[4], animals in which the superior cervical ganglion had been removed were considered to be denervated and animals in which the sympathetic nerve trunk had been sectioned below the superior cervical ganglion (preganglionic section) were considered to have been decentralized.

Since there is already substantial information available on the incidence of iris heterochromia in Horner's syndrome[1,2,3], the current series of animals were prepared primarily for the assays reported in subsequent pages. However, some observations were made on iris color that deserve mention. As already known, after either denervation or decentralization of the sympathetic nerve supply to the eye, rabbits develop a syndrome analogous to Horner's syndrome in the human: the fissure narrows, the pupil is miotic, and there is an apparent enophthalmos. In many of the rabbits a minimal heterochromia is immediately apparent; a heterochromia which is related to variation in iris size, the miotic iris appearing slightly lighter in color than its fellow. If, at this stage, the size of the two irides is made equal by drugs, the color of the two irides is found to be equal.

By barely perceptible degrees the iris on the operated side loses pigmentation. The rate at which this occurs varies from animal to animal. It occurs so gradually that with daily observation it is difficult to date when it is first present. Recognition varies with, among other things, the acuteness of the observer. Often a trained observer can see a difference in iris color at a time before convincing evidence of this difference can be obtained photographically. Thus at three weeks there often is a small but real difference in iris color, a difference which does not record well on photographs but also a difference which does not disappear when the two pupils are made equal in size. By six weeks after surgery the difference in iris color is usually decided enough so that it can be readily photographed in color as well as be perceived by most observers. By four months the difference is pronounced enough that it can even be appreciated in black and white photographs (Figure 3).

Two animals were kept for one year after operation. It was found that there was very little increase in heterochromia over the last eight months of the experiment.

Within the limits of the observations alluded to above, no differences in the development of heterochromia were found between animals that had been decentralized and animals that had been denervated. In this regard it should be noted that four 28-day denervation animals escaped observation for heterochromia. As a consequence, information on the heterochromia of denervation was limited to observation of four animals (5T1, 5T2, 5T3, and 5T7).

METHODS

THE ADRENERGIC INNERVATION OF THE IRIS

The adrenergic innervation of the iris was investigated in a variety of mammalian species, including rat, rabbit, cat, New World and Old World monkey, and baboon. Special emphasis was placed on studies in the baboon. In all animals the innervation pattern was studied by the histofluorometric method for catecholamines.[5,6] In this method, catecholamines such as noradrenalin are converted into highly fluorescent isoquinoline derivatives by exposure to gaseous formaldehyde and heat. In order to prevent diffusion of catecholamine stores during processing, the tissues are first quick-frozen and then freeze-dried at −35°C in vacuum. Once dried and processed, the tissues are embedded in paraffin, the blocks sectioned, and the sections mounted in xylene. When viewed in the fluorescence microscope, adrenergic nerves are uneven, varicose, yellow-green filaments against a dark background.

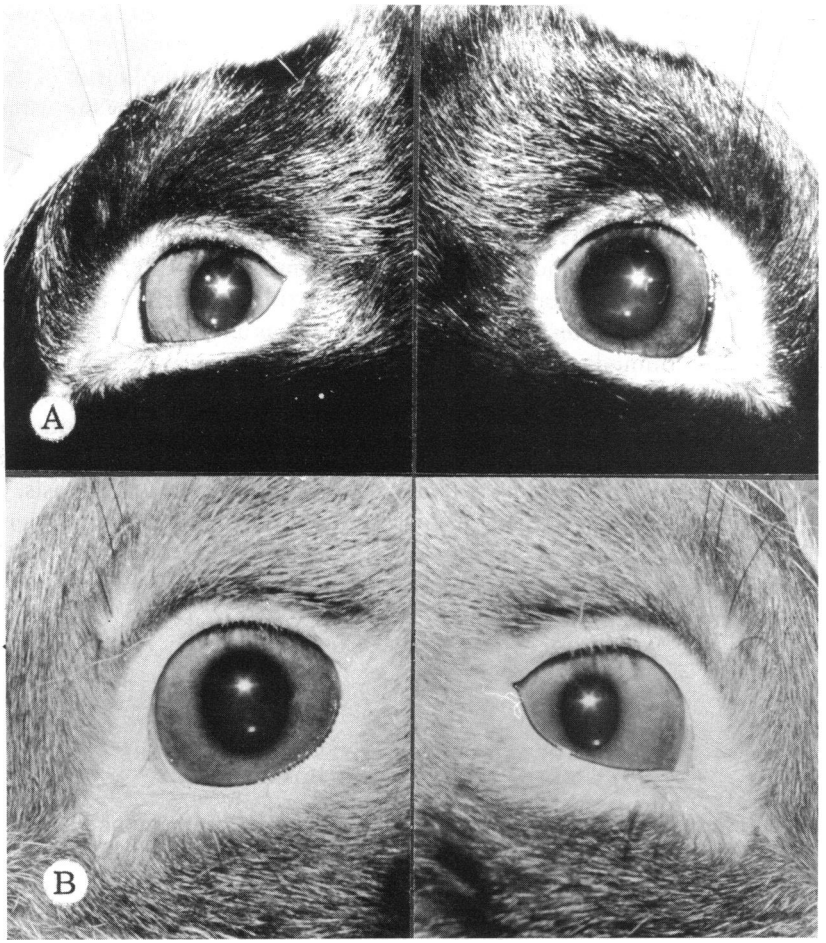


FIGURE 3
Effect of sympathetic decentralization on iris color. Each animal was operated upon on the 4 months before the photographs were taken. Heterochromia is marked in A and slight in B.

TYROSINASE ASSAY

Preparation of Sample: In all instances the procedure followed was the same. Deep barbiturate anesthesia (Nembutal [Abbott Laboratories] 35 mgm/kg intraperitoneal) was induced in Dutch-belted rabbits of either sex, after which one eye was enucleated and placed on ice. The iris was rapidly dissected free and frozen on dry ice. After being weighed it was

stored at below −20°C. The second eye was then processed in a similar manner. In several instances a sample of choroid was also taken.

Assay of Sample: All assays were performed by Dr. Aaron Lerner of the Department of Dermatology, Yale University School of Medicine using the method of Pomerantz.[7]

To accomplish this result, each sample of iris was homogenized at a temperature as close as possible to 0°C in from .3 to .45ml of 0.1M sodium phosphate buffer at pH 6.8. L-dopa 10^{-4} M had been previously added to this solution. After the homogenization, 100 lambda of 2 x 10^{-3} M tritiated tyrosine (1ci/mole) solution was added to each tube, the temperature was raised to 37°C in a water bath and the material was allowed to incubate for 30 minutes. Immediately thereafter, 0.8 ml of HPO_3 was added to each tube to stop the reaction. After the addition of 0.5 ml of a 100 mg/ml suspension of Norit A, each tube was centrifuged and a 0.5 ml aliquot of the supernatant was taken for counting in a liquid spectrometer. Counts from iris samples were calculated on a counts per mg wet weight basis.

Starting with Assay Series 7, the procedure was modified; tissues were no longer homogenized. Rather, whole iris or choroid samples were incubated in 0.5 ml of process solution. Incubation time for iris was two hours, and for choroid five hours.

RESULTS

DESCRIPTION OF CATECHOLAMINE FLUORESCENCE IN THE EYE OF FETAL MACAQUE MONKEY

This description is based on two eyes from a macaque monkey fetus, delivered immediately before term by Caesarian section.

CHOROID AND PIGMENT EPITHELIUM

From the point of view both of autonomic innervation and of choroidal pigmentation, the monkey eye at birth is immature. This is readily appreciated in Figure 4A and B in which the density of uveal melanocytes from a full term macaque fetus is contrasted to those of an adult. Thus, in the macaque at birth, choroidal melanocytes are few in number although in most instances the individual cells have substantial pigmentation. Melanocytes have no typical catecholamine fluorescence within their cytoplasm nor are any neighboring, unpigmented cells with specific catecholamine fluorescence found elsewhere in the choroid.

In the same specimens the adrenergic innervation is sparse (Figure 5C and D). Of the adrenergic nerves seen in the choroid, the largest number

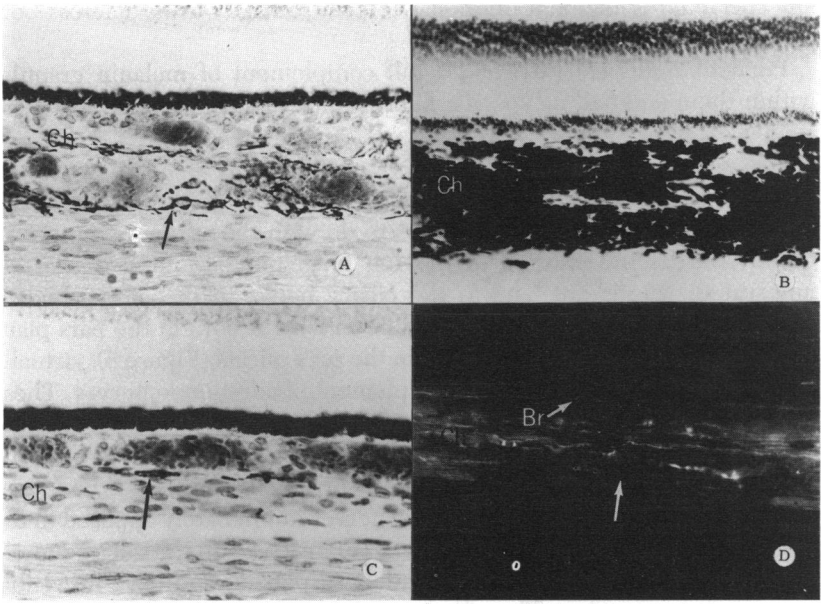


FIGURE 4

Comparison of density of melanocytes (arrows) in monkey fetal choroid (Ch) at term (A) with that in the adult (B). There is also a regional variation in the density of choroidal melanocytes in the fetus. Compare A with C; both microphotographs are of the same tissue section (Luxol blue-cresyl violet stain, magnification 4A - × 192; magnification 4B - × 155). (C) Near ora serrata melanocytes are few (arrows) and are widely dispersed. (D) Varicose fluorescent nerves (black arrow) for the most part have no apparent relationship to the melanocytes (white arrow). Bruch's membrane (Br arrow) is autofluorescent and clearly separates choroid (Ch) from retina (4C-Luxol blue-cresyl violet stain magnification × 273; 4D-Histofluorometric method, magnification × 397).

are in apparent apposition to blood vessels or run free. On occasion, an adrenergic nerve passes close enough to a melanocyte to raise the question whether or not such a melanocyte could be innervated. This question cannot be answered from the slides prepared for this study. Choroidal melanocytes are in general oriented with their long axis running parallel to Bruch's membrane.

Bruch's membrane is always highly visible (see Figure 4D); it is brilliantly autofluorescent and clearly delimits the retina from the choroid. In no instance was an adrenergic nerve seen to cross Bruch's membrane to

innervate the retinal pigment epithelium. Nor were there any signs in any specimen of pigment epithelial cells in passage outward across both membranes to the choroid.

Pigment epithelial cells had a full complement of melanin granules within them and had no specific fluorescence in their cytoplasm.

CILIARY BODY

There are substantial differences between the choroid and the ciliary body, the most marked of which has to do with the adrenergic innervation. Whereas the choroid of the full term monkey fetus is sparsely innervated, this is not true in the ciliary body. In the ciliary body a plentiful basal adrenergic plexus can be made out both in the pars plana and in the pars plicata. It is densest in the pars plicata (Figure 5); virtually each ciliary process receives its complement of adrenergic nerves. These nerves are confined to the stromal part of each process and are not seen to enter between epithelial cells. It should be noted, however, that over the anterior portion of the pars plana where Bruch's membrane is no longer visible, it is possible to see adrenergic nerves immediately apposed to the basal surface of pigment epithelial cells (Figure 6A). In the ciliary muscle proper, melanocytes are few, are widely dispersed, and have no visible adrenergic innervation. The ciliary muscle itself is also devoid of nerves save for an occasional adrenergic twig about a blood vessel. Some adrenergic nerves, preterminal in fluorescence characteristic, are visualized in sections containing the long posterior ciliary nerve.

The pigmented epithelium of the ciliary body, both in the pars plana and the pars plicata, appears mature; these cells have numerous melanin granules within their cytoplasm and no specific catecholamine fluorescence. In contrast, the ciliary epithelium has neither melanin granules nor catecholamine fluorescence. No adrenergic nerves are visualized between any epithelial cells in the ciliary body.

THE IRIS

Of the three divisions of the uveal tract, the iris seems to be the most mature; at least, if maturity is measured by number of stromal melanocytes and the density of their pigmentation. Stromal melanocytes are most abundant in the anterior border layer near the collarette. On rare occasions an adrenergic nerve can be seen in apparent apposition to an iris stromal melanocyte; however, for practical purposes it can be said that they are devoid of innervation; for large masses of iris stromal melanocytes are visualized among which no adrenergic nerves at all are found.

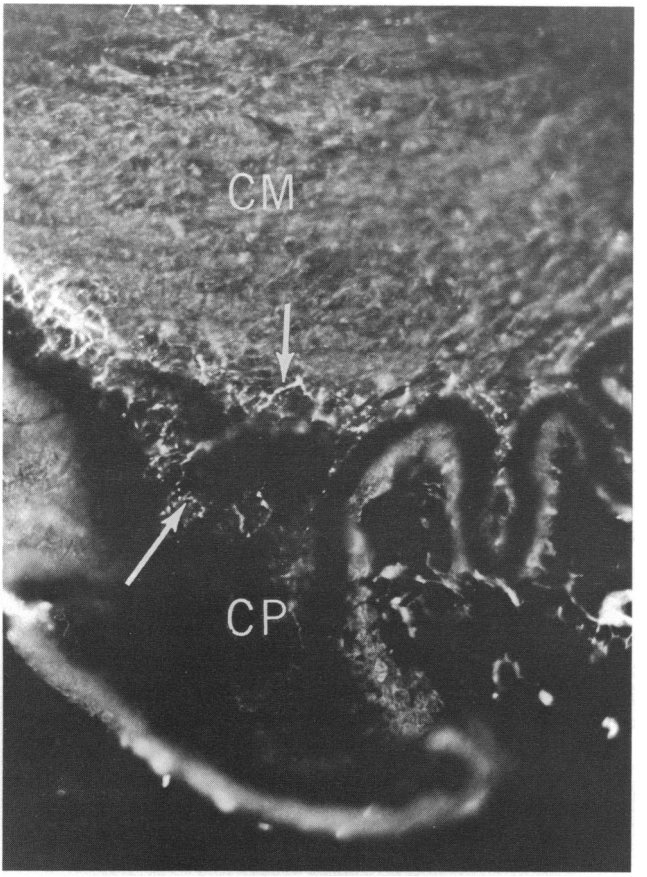
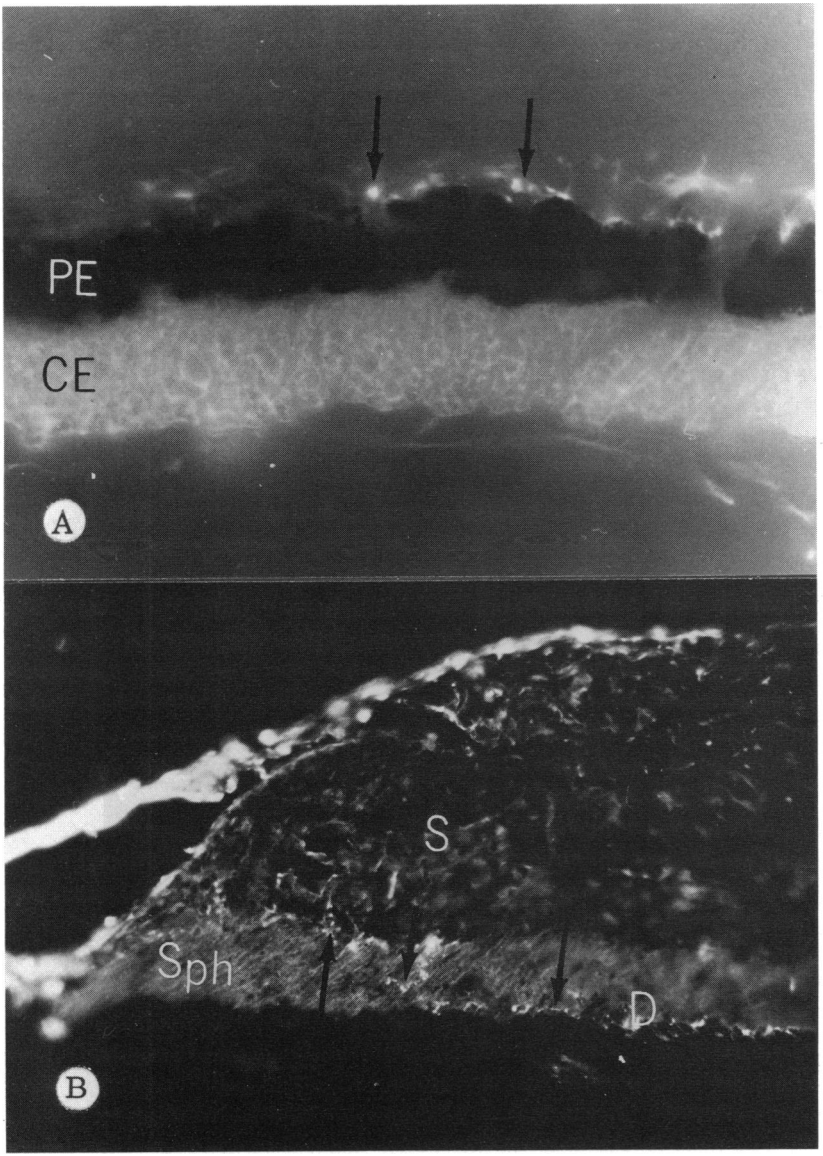


FIGURE 5

Term macaque monkey fetus. Fine, varicose, adrenergic nerves (arrows) are present in each and every ciliary process (CP). The pigmented epithelium is non-fluorescent. No nerves are visible in the ciliary muscle (CM). (Histofluorometric method, magnification × 250).


PE
CE
A
S
Sph
D
B

(opposite) FIGURE 6

A: Term macaque monkey fetus. Many varicose, adrenergic nerve fibers (arrows) are found immediately adjacent to the pigmented epithelium (PE) of the pars plana. Ciliary epithelium (CE) is visible by its autofluorescence. No specific fluorescence is visible in either epithelial layer (Histofluorometric method, magnification × 970). B: Term macaque monkey fetus. At term the iris sphincter is formed and is visible because of autofluorescence. In this preparation numerous adrenergic nerves are present in the iris dilator (D) region. In addition, some varicose nerve fibers (arrows) are also present within the sphincter muscle (Sph) and in the iris stroma (S) immediately adjacent to it. No adrenergic fibers are seen in the iris stroma itself although many melanocytes are present. Note autofluorescent band on anterior surface of iris (Histofluorometric method, magnification × 243).

Iris stromal melanocytes also do not have catecholamine fluorescence within their cytoplasm. In short, in this aspect at least, they seem to be mature.

In the same tissue sections the adrenergic innervation is plentiful in the region of the dilator muscle, where it forms a fine, dense filigree just in front of the anterior epithelial cells. This network of nerves may be traced from the iris root where large preterminals stream inward toward the pupillary rim. As a general rule, the adrenergic innervation in the dilator region begins to thin out as it passes beneath the sphincter muscle (Figure 6B).

There are also a modest number of adrenergic nerves visible within the sphincter muscle itself and in the iris stroma to either side of it. On occasion a few fluorescent nerves run forward from the anterior surface of the sphincter into the iris stroma (Figure 6B).

In many sections, an unusual type of pigmented cell is also visible. These cells are autofluorescent. That is, they fluoresce both in the experimental slides and in control slides (tissues not exposed to paraformaldehyde vapor). In a few sections these cells formed a continuous lining on the anterior surface of the iris, extending from the pupillary rim to the collarette. At times, they were quite large and contained numerous melanin granules within their cytoplasm (Figure 7). Further, similar cells are on occasion seen on the endothelial surface of the cornea, on the surface of the trabecular meshwork, or on the posterior surface of the iris.

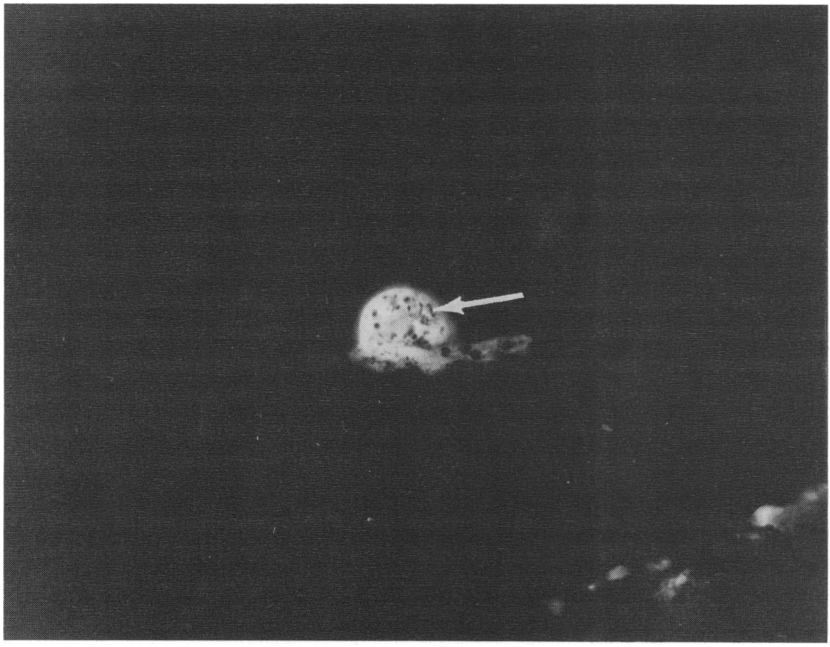


FIGURE 7
Detail of cell on anterior surface of iris of macaque fetus at term. Note melanin granules (arrow) within its cytoplasm (Histofluorometric method, × 640).

Description of Catecholamine Fluorescence in the Developing Eye of the Rat. Term Fetus and Days 1 and 2

OPTIC CUP DERIVATIVE

There are remarkable differences in the intensity of specific catecholamine fluorescence in the neuroepithelial layers; all specific fluorescence is confined to the outer, pigmented epithelium. Within this layer there are again remarkable differences in fluorescence intensity that have to do with location. Specific fluorescence is intense at the ora serrata, at the ciliary body, and at the root of the iris (Figure 8). Fluorescence intensity declines gradually as the pigment epithelium of the retina is traced

posteriorly. However, even at the posterior pole, specific fluorescence is still visible. There is some variation from specimen to specimen in the extent to which the anterior epithelium of the iris fluoresces. However, in most specimens fluorescence is maximum at the iris root and lessens dramatically at the junction of the outer and middle thirds. Yet minimal to trace amounts of fluorescence are visible in the anterior pigment epithelial cells of the iris all the way to the developing sphincter. But only trace amounts of specific fluorescence are visible in the developing sphincter muscle itself, despite the obvious presence of cytoplasmic pigment granules. No specific fluorescence is visible in the cells of the posterior pigmented layer of the iris.

When the fluorescence intensity in pigmented epithelial cells is compared to pigment content of the same cells, there is not at this stage a clear correlation between the two; some pigment is present in all cells derived from the outer layers of the optic cup.

THE MESODERMAL LAYERS

The mesenchymal tissues are very primitive and lag behind the epithelial tissues in differentiation (Figure 8). Thus, a distinct choroid is not visible and the ciliary body and iris are both rudimentary. In fact, the iris is really just a short, peripheral stub in which the mesodermal and epithelial components retain a separate identity (Figure 9).

Even at this stage, iris stromal melanocytes are numerous. In bright field sections they are readily visible (Figure 10). They extend as a band from the region of the bulbous early sphincter, past the iris root in a backward-bowed arc and continue into the developing ciliary body. Individual cells are oriented more or less with their long axes parallel one to another and parallel to the outer coat of the eye. As the iris stromal melanocytes blend into the ciliary body, their numbers decrease remarkably.

At this stage of development melanocytes are far less numerous in the primitive choroid than in the iris. The choroidal melanocytes form a discontinuous and scattered layer, a layer separated by some distance from the pigment epithelium. In no instance was there evidence of any choroidal melanocyte being immediately adjacent to the pigment epithelium, nor was there any evidence of pigment epithelial cells migrating outward. Individual choroidal melanocytes appear to have numerous pigment granules within them.

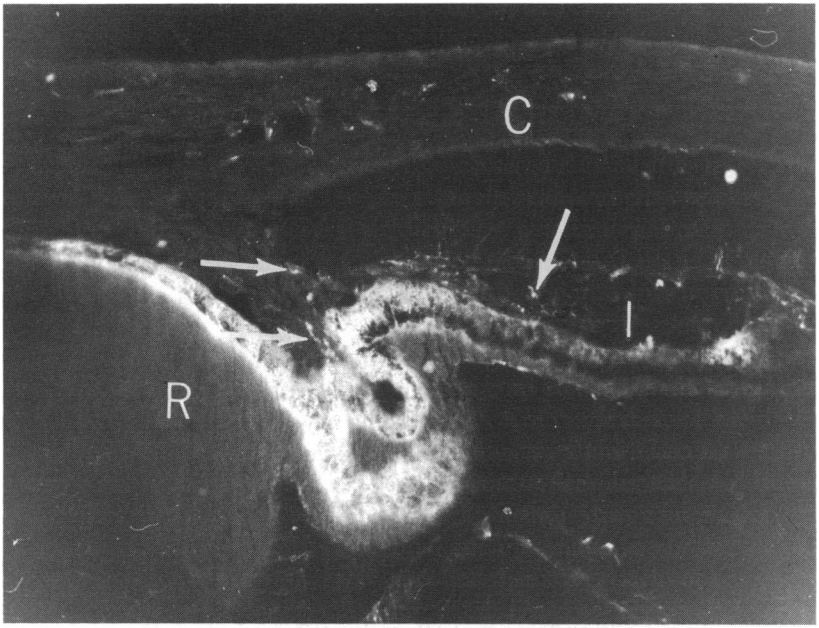
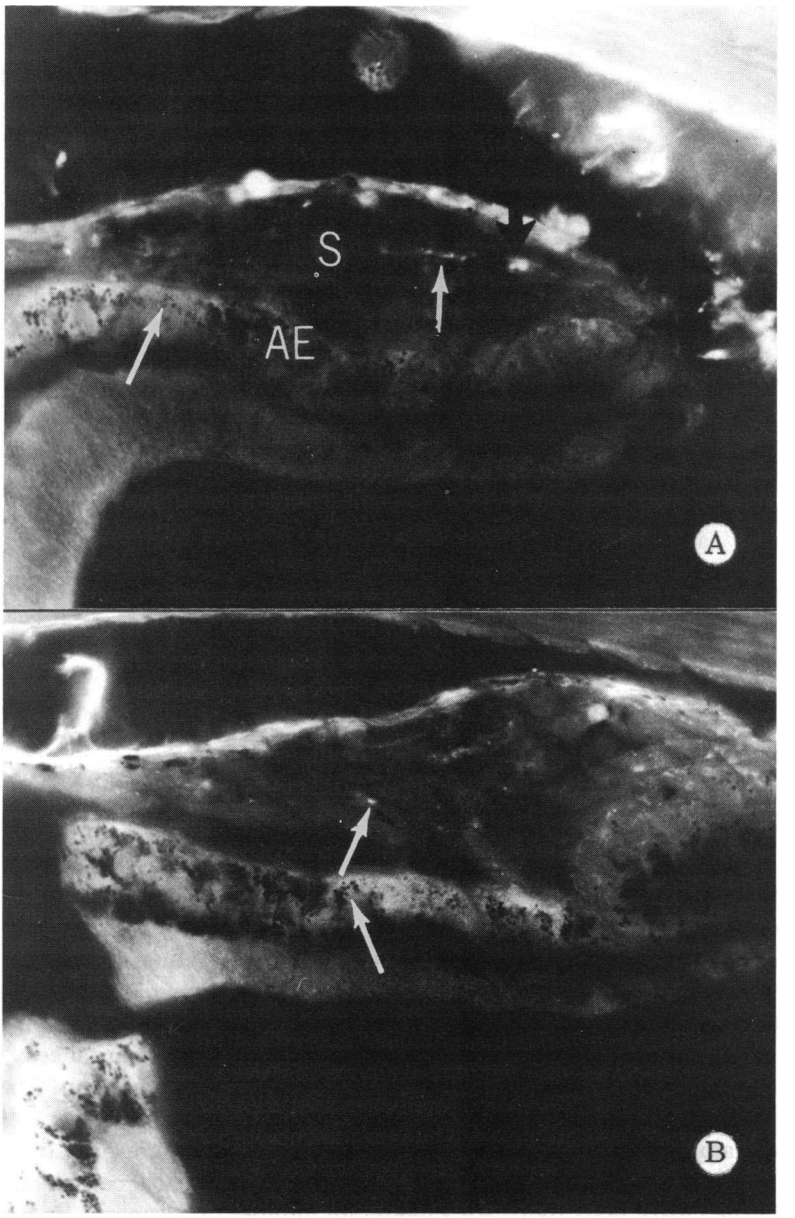


FIGURE 8

New-born pigmented rat. Low power, fluorescence microphotograph demonstrates presence of a small number of adrenergic nerve fibers (arrows) in developing ciliary body and iris (I). Cornea (C) is visible at top of picture. Brilliantly fluorescent pigment epithelium of the retina (outer layer of optic cup) continues forward as epithelium of ciliary body without discernible change in fluorescence intensity. Retina (R) at ora serrata is still actively differentiating (Histofluorometric method, magnification × 255).

FIGURE 9 *(opposite)*

At term the epithelial and mesodermal components of the rat iris are clearly distinguishable. Anterior pigmented epithelial cells (AE) (arrows) are fluorescent. Several varicose, adrenergic nerve fibers (arrows) are visible in iris stroma (S). At times it is not possible to tell a developing melanocyte from an adrenergic nerve fiber (thick black arrow) (Histofluorometric method, magnification × 602).


S
AE
A
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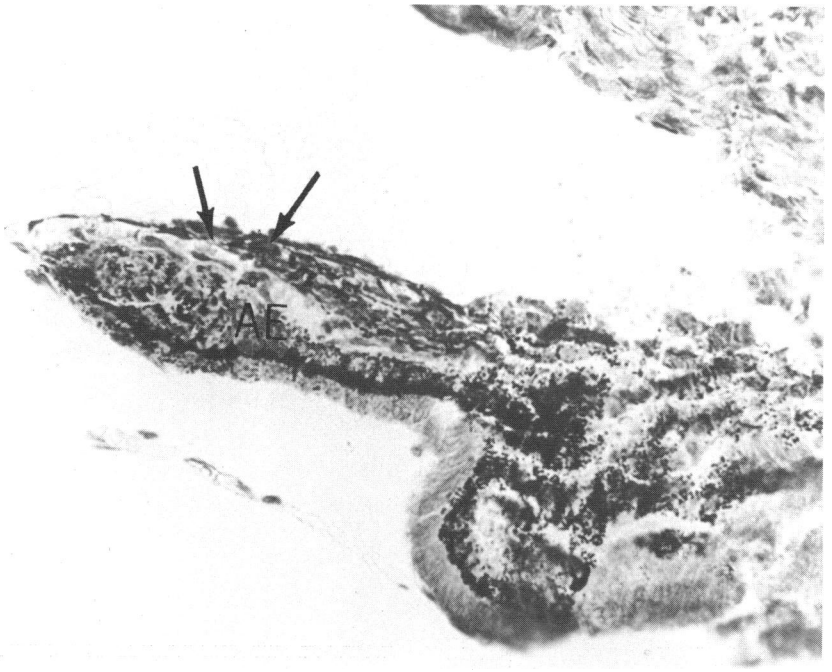

FIGURE 10
Low power, brightfield view of iris and ciliary body of new-born pigmented rat. Some pigmented iris stromal melanocytes are already present (arrows). Also note that anterior epithelium (AE) of iris is already pigmented while pigmentation of the posterior layer is just beginning (Luxol blue-cresyl violet stain, magnification ×310).

THE ADRENERGIC INNERVATION

There are fluorescent, adrenergic fibers in the uveal tissues of each tissue section studied. However, their relationship to melanocytes is not apparent. For instance, whereas an adrenergic terminal can be visualized on occasion just next to an iris stromal melanocyte, in most instances adrenergic nerves are found to be without obvious relationship to any other cellular structure in the iris. The one exception to this statement is in the iris dilator region where a few fluorescent nerve fibers are present and where their immediate relationship to the anterior epithelial cells is clear.

In the ciliary body there are a moderate number of adrenergic nerves, but again the nerves are without obvious relationship to melanocytes. In

the choroid, melanocytes are very few and adrenergic nerves are few; there is no obvious relationship between the two.

Despite the active proliferation of uveal melanocytes taking place, there is no obvious counterpart to the brilliant intracellular fluorescence characteristic of the pigmented epithelial cells. In sharp contrast, most uveal melanocytes do not fluoresce. A rare fluorescent cell probably containing catecholamine is also seen in choroid or in ciliary body. Exceptions to this statement are few. The newly-forming iris especially in its anterior border region may be an exception in that some cells with fluorescent cytoplasm are visible. However, the presence of a layer of autofluorescent* cells at the anterior surface of the iris partially beclouds the issue in this region and makes evaluation difficult (see Figure 9).

DAYS 4 TO 7

The iris has grown substantially and has assumed a mature form (Figure 11A and B). Both epithelial layers now contain pigment granules, although even at Day 6 the pigment is still less dense in the posterior than in the anterior epithelial layer. There are a multitude of iris stromal melanocytes now present, the greatest density of these cells being near the anterior border layer. The innervation pattern of the iris has changed little in the last few days. What nerves there are are scattered and without apparent relationship to melanocytes. Adrenergic nerves differ somewhat in location from tissue section to tissue section but are most commonly found in the region just in front of the iris sphincter.

Change is rapid in the developing posterior segment. Substantial melanosome proliferation has taken place in the pigment epithelium at the ora serrata (Figure 12A). At Day 4 melanocytes tend to be visible in two locations: just at the ora serrata where they are in reality a spillover of the stromal melanocytes of the ciliary body and at the back of the eye. In each instance the cells are not fluorescent and are oriented with their long axes running parallel to the sclera. At the equator choroidal melanocytes are rarely seen. However, by the end of Day 6 choroidal melanocytes are now present in the equatorial region and melanocytes therefore form a nearly continuous band from posterior pole to ora serrata. Even now adrenergic nerves are scattered and few; those that are visible are present mainly in the posterior pole and in that region for the most part are draped about blood vessels. It is rare to see an adrenergic nerve near a choroidal melanocyte.

*For discussion of this cellular layer in foetal life, see F. Vrabec, Reference 8.

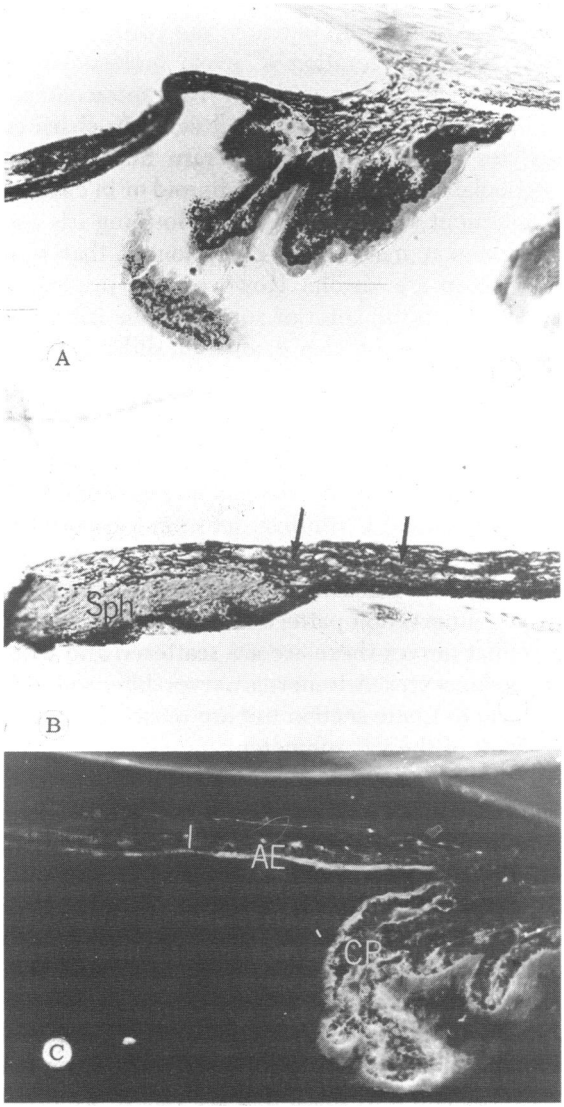

FIGURE 11

By 7 days of age there has been substantial development of the iris and ciliary body, as illustrated in Figures 11A and 11B. Note density of iris stromal melanocytes (arrows). At the same time there are still very few adrenergic nerves in the iris stroma. C: Specific fluorescence of anterior epithelium (AE) of the iris (I) persists as does that of the pigment epithelium of the ciliary process (CP) (11A and B-Luxol blue-cresyl violet stain; 11C-Histofluorometric method; magnification × 168).

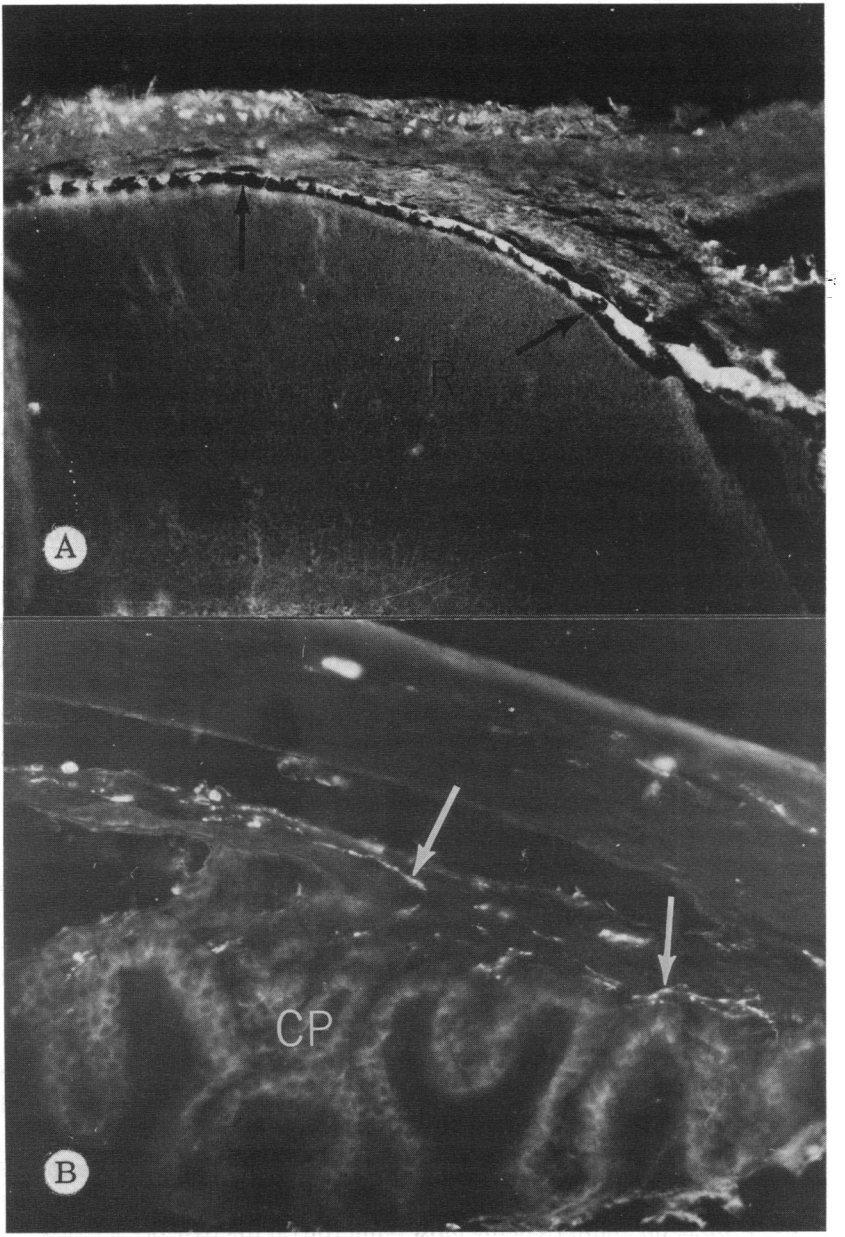
A
B
CP

FIGURE 12 *(overleaf)*

A: Development of pigment epithelium in a 4 day old rat. In comparison to the newborn eye illustrated in Figure 8, there has been a remarkable increase in melanin granules (arrows). However, considerable cytoplasmic fluorescence persists (Histo-fluorometric method, magnification × 250). B: 11 day old albino rat. Catecholamine fluorescence is present in the basal epithelial layer of the ciliary processes (CP). Several varicose adrenergic nerve fibers (arrows) are present in ciliary body and some are entering the iris (Histofluorometric method, × 250).

DAYS 12 TO 16

There has been a remarkable development of the adrenergic innervation of the iris (Figures 12B and 13). A network of preterminal and terminal nerve fibers now innervate large regions of the iris stroma. Fine varicose terminals ramify among the iris stromal melanocytes and often are in direct apposition to them.

No specific fluorescence is any longer visible in the iris epithelial layers. However, specific fluorescence persists in the pigmented epithelium of the ciliary process (Figure 12B).

In the posterior segment specific fluorescence of the pigment epithelium has largely disappeared, save for faint residual fluorescence just at the ora serrata. Choroidal melanocytes are now plentiful. They are mainly located in the outer two-thirds of the choroid. None are seen in position to support a contention that they derive in any way from the pigment epithelium of the retina. Adrenergic nerves are present in moderate density. By far the largest number of them are directly apposed to blood vessels.

ADULT PIGMENTED RAT

In the iris of the adult rat a substantial innervation is clearly present. Iris stromal melanocytes are so dense and so numerous that they make visualization of adrenergic nerves difficult at times. However, the two are frequently in close approximation to each other (Figure 14). Fluorescent varicose nerves can be seen (a) as a scattered network in the iris dilator region, (b) as a scattered network near the anterior border layer of the iris, and (c) in the region of the sphincter muscle (in this case both in it and to either side of it, anterior or posterior). There is no fluorescence whatever of the pigment epithelial layers of the iris.

The ciliary processes no longer have any specific fluorescence in the pigmented epithelium. There are apparent differences in melanin granule density, melanin granules being most numerous at the base of each process and least numerous at the crest.

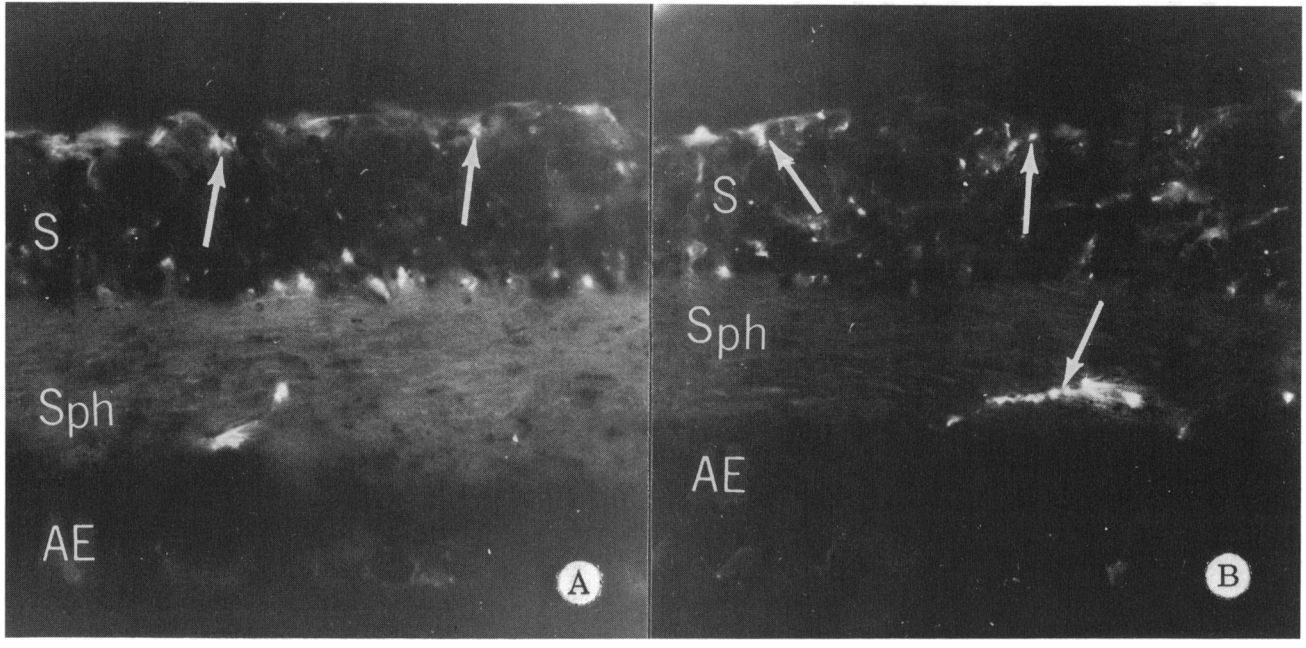

FIGURE 13

Two illustrations of the extent of the adrenergic innervation of the iris at 15 days. Varicose, fluorescent nerve fibers (arrows) are now present in iris stroma(S). They often congregate in anterior border layer. On occasion, one is seen within the substance of the iris sphincter (Sph). Anterior epithelium (AE) is just perceptible in darkfield microscope (Histofluorometric method, magnification × 640).

In the posterior segment there is no catecholamine fluorescence visible in the pigment epithelium, and all pigment epithelial cells have a substantial number of melanosomes within them. The melanosomes tend to aggregate in the half of the cell nearest the photoreceptor and are elliptical in form; most appear to be oriented with their long axis parallel to the long axis of the photoreceptor outer segments. In addition, a large number of minute, orange-fluorescent inclusions are now visible in the pigment epithelial cells.

The situation in the choroid is still not entirely clear. There is no question but that the large blood vessels of the choroid are heavily innervated by adrenergic nerves. The choroidal melanocytes, however, remain a puzzle. In selected areas, adrenergic fibers can be seen in apposition to them. However, in most areas this is not the case; for great numbers of melanocytes are visible with only an occasional adrenergic fiber among them. The same statement holds true for melanocytes in the ciliary body in the same preparations.

No adrenergic nerves are ever seen in any preparation to leave the choroid to enter the pigment epithelium of the retina.

HISTOFLUOROMETRIC RESULTS IN OTHER SPECIES

In all species studied, a high density of adrenergic innervation blankets the entire extent of the iris dilator muscle. Although in close apposition to the pigment epithelium of the iris, no fluorescent fibers are seen between or in individual epithelial cells. The sphincter muscle of the iris also receives some adrenergic innervation in all species. The density of adrenergic innervation to the sphincter muscle varies from a low in the primate to a high in the cat.

Iris stromal melanocytes have widely varying appearances among different species. In the fluorescence microscope they vary from dull brown in many species to a brilliant yellow in the cat (hence called chromatophores). In all species studied, some varicose adrenergic nerves are seen immediately apposed to iris stromal melanocytes. Usually the varicose fibers are of highest density about melanocytes located near the anterior border layer of the iris. As illustrated in Figure 15 of the baboon iris, the number of such contacts can be substantial. In this microphotograph a myriad of varicose, fluorescing nerve fibers surrounds reddish, autofluorescent melanocytes in the anterior border layer in the region of the collarette. In contrast, the density of innervation of iris stromal melanocytes is lower in all other species studied; in cat, for instance, it is necessary to search carefully to find nerve-melanocyte or nerve-chromatophore contacts at all.

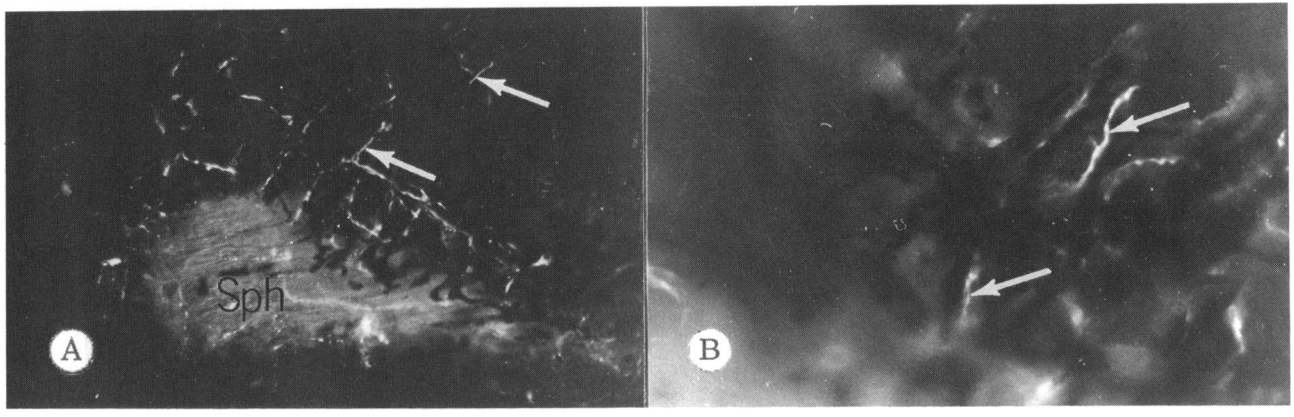

FIGURE 14

Numerous, fine adrenergic nerve fibers (arrows) are present in iris stroma just in front of the sphincter muscle (Sph). Under high power, it is apparent that the nerve fibers (arrows) are immediately adjacent to iris stromal melanocytes (Histofluorometric method; A-magnification × 240; B-magnification × 614).

As a check on the specificity of the histochemical method used, iris tissues from two rabbits and two cats were processed for catecholamine fluorescence 3 to 6 days after removal of the superior cervical ganglion. In these tissues no fluorescent adrenergic nerves whatsoever were present in the iris.

RESULTS OF TYROSINASE ASSAYS

A complete listing of the results of the iris assays is presented in Table I, Parts A and B; included are notations on iris color before operation, on coat color, on the duration of the experiment and on whether or not heterchromia developed.

The chief results are then highlighted in Tables II to VI. To bring some order out of variation in the level of tyrosinase activity from animal to animal and out of what are essentially artificial standards for the measurement of tyrosinase, standards which change on occasion during the experimental series, all measurements are expressed in comparative terms;

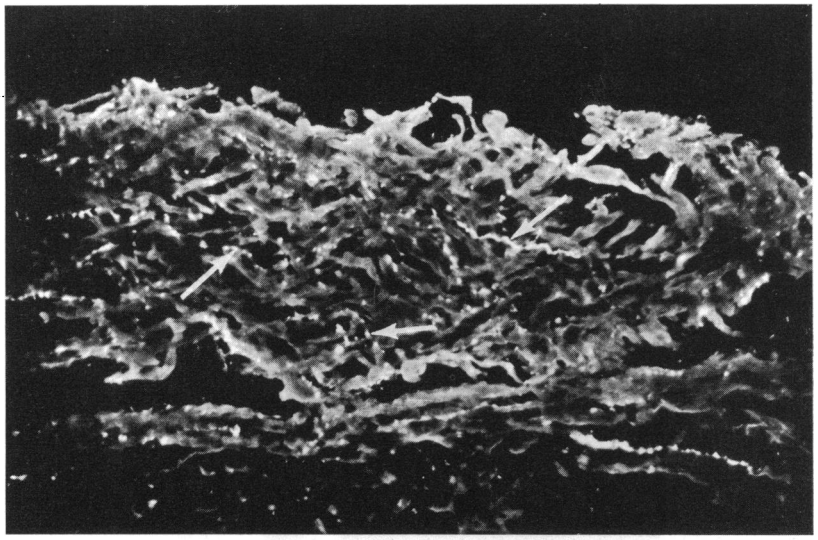

FIGURE 15
Iris of adult baboon. Arrows point out some of the numerous adrenergic nerve fibers (Histofluorometric method, magnification × 225).

TABLE 1A: DENERVATION SERIES

Experiment	Days Survived After Operation	Coat Color	Iris Color	Iris Heterochromia	Tyrosinase Operated Side as % of Control Side
4-21	1	Br, Bl & W	Brown	- - -	59.0
4-13	4	Bl & W	Brown	- - -	61.9
4-22	28	Br & W	Brown	Not Evaluated	62.0
4-23	28	Br & W	Brown	Not Evaluated	87.7
4-24	28	Br & W	Brown	Not Evaluated	52.6
4-25	28	Bl & W	Brown	Not Evaluated	74.0
5-T1	105	Bl & W	Brown	++ Obvious 7 wks. after operation	77.5
5-T2	105	Bl & W	Brown	+ "	44.8
5-T7	105	Br & W	Brown	++ "	81.1
5-T3	370	Grey & White	Grey-Brown	+ "	101.0

TABLE 1B: DECENTRALIZATION SERIES

Experiment	Days Survived After Operation	Coat Color	Iris Color	Iris Hetero-chromia	Tyrosinase: Operated Side as % of Control Side
2586-9	1	Grey & W	Grey	0	61.8
2587-9	1	Bl & W	Brown	0	76.9
2588-9	1	Bl & W	Brown	0	77.0
2589-9	1	Bl & W	Brown	0	60.7
2580-9	4	Bl & W	Dk. Brown	0	56.9
2581-9	4	Bl & W	Brown	0	82.2
2582-9	4	Bl & W	Brown	0	49.6
2583-9	4	Br & W	Lt. Brown	+	58.6
2570-8	35	Bl & W	Dk. Brown	++	69.7
2567-8	42	Bl & W	Dk. Brown	++	91.7
2568-8	42	Grey & W	Grey	++	96.4
2565-8	56	Taffy & W	lt. Brown	++	53.4
2566-8	56	Bl & W	Dk. Brown	+	43.0
2569-8	56	Dun & W	Brown	++	77.8
5-T9	140	Bl & W	Brown	++	32.7
5-T6	161	Bl & W	Brown	++	96.0
5-T8	161	Br & W	Brown	++	74.6
2576-9	280	Bl & W	Brown	++	55.0

TABLE II: EFFECT OF INTERRUPTION OF SYMPATHETIC PATHWAY ON IRIS TYROSINASE LEVEL

	CONTROL SERIES Right Eye as % of Left Eye	ALL OPERATED ANIMALS Operated Side as % of Control Side
MEAN	96.4	68.3
STANDARD DEVIATION	26.3	17.4
STANDARD ERROR	5.2	3.3

Difference between means of Control and Experimental Significant, $P < .0005$

TABLE III: EFFECT OF SYMPATHETIC DENERVATION ON IRIS TYROSINASE

	CONTROL SERIES Right Eye as % of Left Eye	DENERVATION SERIES Operated Side as % of Control Side
MEAN	96.4	70.0
STANDARD DEVIATION	26.3	17.1
STANDARD ERROR	5.2	5.4

Means are Significantly Different, $P < .005$

TABLE IV: EFFECT OF SYMPATHETIC DECENTRALIZATION ON IRIS TYROSINASE

	CONTROL SERIES Right Side as % of Left Side	DECENTRALIZATION SERIES Operated Side as % of Control Side
MEAN	96.4	67.4
STANDARD DEVIATION	26.3	18.1
STANDARD ERROR	5.2	4.2

Means are Significantly Different, $P < .001$

experimental results are expressed as level of tyrosinase in the experimental iris as a percent of that in the control iris. Since right and left irides of each animal were always processed in the same assay run; had the same incubation time; the same incubation temperature; the same process solution, etc.; the results were freed from some of the vagaries inherent in

successive biochemical assays. For the control series, results are expressed in terms of left iris divided by right iris.

The most important results as presented in Tables II to VI are as follows: Tyrosinase activity in the iris of the pigmented rabbit falls rapidly after interruption of the sympathetic pathways to the eye; once diminished, enzyme activity settles permanently to a new level and remains more or less at that level in succeeding months.

In Table II, a pooled sample of all operated animals is compared to a control population. The difference between the mean of the two populations is substantial and is statistically significant.

The significance of the pooled results is not lessened in a biologically meaningful way if the experimental populations are divided into two logical sub-groups, (1) those animals in which the superior cervical ganglion had been extirpated (denervation) and (2) those animals in which preganglionic section of the sympathetic trunk in the neck had been performed (decentralization). As presented in Tables III and IV, each of these populations, although of modest size, differs in a significant manner from the control population.

TABLE V: IRIS TYROSINASE LEVELS: DENERVATION VERSUS DECENTRALIZATION

	DENERVATION SERIES Operated Side as % of Control Side	DECENTRALIZATION SERIES Operated Side as % *of* Control Side
MEAN	70.0	67.4
STANDARD DEVIATION	17.1	18.1
STANDARD ERROR	5.4	4.2
	Means are not Significantly Different	

TABLE VI: EFFECT OF SYMPATHETIC DECENTRALIZATION ON CHOROID TYROSINASE

	CONTROL SERIES Right Eye as % of Left Eye	DECENTRALIZATION SERIES Operated Eye as % of Control Eye
MEAN	100	77
STANDARD DEVIATION	30	15
STANDARD ERROR	8	4
	Means are Significantly Different, $P < .01$	

It is also of interest to compare the two operated populations one to the other. As shown in Table V, the two populations are indistinguishable, differing by less than one standard error of the mean from each other. It is the closeness of the results that justifies pooling the data on all operated animals and the subsequent comparison of the pooled and control group in Table II.

An attempt was also made to analyze the assay results in terms of iris color and of duration of experiment. For each evaluation, the number of animals in individual categories was small as were the differences. Thus, there were no changes in level of tyrosinase activity that could be related to original color of the iris, nor were there any significant differences between the results of experiments of short duration when these were compared to experiments of long duration.

Changes in tyrosinase levels as a function of original pigment density or of duration of the experiment either are not present, or, if present, are of insufficient magnitude to be significant in samples as small and as variable as those presented.

As a further and somewhat different check on the relationship of eye color to iris tyrosinase, tyrosinase assays were done on the irides of two Dutch-belted rabbits which were spontaneously heterochromic. In each of these two instances there was a marked difference in the assays, the lighter colored iris having less tyrosinase activity than the darker one.

TYROSINASE LEVELS IN CHOROID

Assays of choroidal tyrosinase were undertaken in systematic fashion only on the decentralization series. They differ in significant fashion from a control group. Just as was the case for the iris, the adrenergic innervation clearly appears to have a role in the regulation of choroidal tyrosinase levels.

When the 16 assay results from the experimental animals are plotted as a function of time, there is an intriguing downward drift of the means, from the first readings at one day to the last reading at six months. However, the differences in such small groups are not significant to the student t-test.

TYROSINASE ACTIVITY: CHOROID VERSUS IRIS

In performing the assays, it quickly became apparent that choroid has substantially greater tyrosinase activity on a per mgm wet weight basis than does iris. Some measure of this difference in activity is given by the difference in incubation times in process solution finally adopted, two hours for choroid and five hours for iris. In a few instances, when iris and choroid had similar incubation time the choroid gave readings 8 to 20 times greater than the iris.

DISCUSSION

As is so often the case, scientific results have a way of surprising those who seek them. At least for the present series of studies, this statement is apt. What was meant originally to be a severely restricted focus, a focus limited to the relationship of adrenergic nerves to iris stromal melanocytes, broadened out to encompass special aspects of chemical embryology and in some instances general neuropharmacology. Thus, in the light of the laboratory results reported here, it is necessary to discuss not only the question of innervation of melanocytes, but also differences to receptor structures between complete loss of innervation (denervation) and innervation by autonomic nerves from a ganglion bereft of its central connection (decentralization); some aspects of the mechanism of melanin formation within cells; and some of the similarities and differences between melanin-containing cells of neuroepithelial origin and those of neural crest derivation. Lastly, the relevance of Horner's syndrome to iris heterochromia is discussed in terms of the laboratory results.

First, a word in justification of the methods used. For the histofluorometric method for biogenic amines a substantial body of information is now available concerning both specificity and sensitivity. First of all, it has been shown in model systems[9] that under the conditions used catecholamines such as l-dopa, dopamine, noradrenalin, and adrenalin form highly fluorescent quinoline structures. Not only has the general nature of the sequence of chemical reactions that take place when catecholamine is exposed to formaldehyde gas been worked out, but the fluorescence characteristics of several of the isoquinoline compounds formed in this reaction have also been analyzed.[9,10]

Perhaps no proof of specificity is as powerful as the biological ones available; and among them, none is so convincing as is the result of surgical denervation. For it has been repeatedly shown that within a few days after removal of the appropriate sympathetic ganglion, the histofluorometric method no longer permits visualization of any adrenergic nerve fibers whatsoever.[11,12] When ultrastructural analysis of similarly denervated tissues has been undertaken, the fact that the adrenergic nerve network undergoes dissolution has been confirmed.[13,14] In a similar vein, pharmacological treatments which deplete tissues of catecholamine stores also lead to a null result with the method; after full reserpinization, for instance, no adrenergic nerve fibers whatsoever can be visualized in the eye by the histofluorometric method.

The sensitivity of the histofluorometric method is also remarkable. Dependent as it is on the visualization of fine, brilliant structures against a

black background, and fortunate in the fact that the fluorescent structures are in high local concentration, the histofluorometric method allows definition of quantities as minute as 0.4×10^{-6} micrograms in the cytoplasm of a neuron, or, more amazing still, 5×10^{-9} micrograms in a varicosity of a peripheral adrenergic terminal.

OCULAR PIGMENTATION AND ADRENERGIC INNERVATION

The observations reported in this paper on the relationship of the developing adrenergic innervation in the eye of rat and monkey to newly-forming uveal melanocytes, and the observations previously reported by Ehinger and Sjoberg[16] on human and guinea pig fetal eyes together support two conclusions: (1) pigment production in uveal melanocytes, especially those of the iris, generally precedes innervation rather than the reverse; (2) some level of adrenergic innervation is present, even if it is immature and not in apparent connection with any receptor structure, during the period when melanocytes are developing.

Thus, it is clearly shown in Figure 6B of the fetal macaque monkey eye that a substantial number of iris stromal melanocytes are present at a time when the innervation of the iris stroma is scant.

Moreover, there is the likelihood that even when adrenergic nerves are visible in developing animals they may not be functional. This question has been studied in detail by Burnstock and his group[17,18] in the mouse vas deferens. This tissue offers many of the same advantages that the rat eye offers for the study of development. In each animal the tissue is immature at birth. It is therefore possible to study with accuracy and with facility the maturation of a given tissue, free of the various difficulties which attend work with embryos. Of immediate pertinence are the twin observations that nerve fibers containing catecholamines could be seen by the fluorescence histochemical technique and dense-cored vesicles could be visualized with the electron microscope; both at a time in the developing vas deferens when the smooth muscle that would normally be subject to a nerve stimulus was incompletely formed and at a time when the muscle is not yet able to respond. Thus, apparently mature, varicose adrenergic nerves are visible at fifteen days of age, but excitatory junction potentials after nerve stimulation are not recordable in muscle cells until eighteen days after birth. By analogy the mere presence of varicose adrenergic nerves in the iris, even if it is accepted that they contain substantial levels of adrenergic neurohumor, does not indicate that a functional system is in being. The receptor may simply not yet be in working condition.

The question remains whether adrenergic nerves might not still have some influence over the developing iris stromal melanocytes at a time that they are not part of a mature, functional unit; in short, whether they might not play an inductive role. The present study contributes no information to this problem. However, the problem is still important as there are clearly documented cases of congenital heterochromia of the iris in instances of congenital Horner's syndrome;[1] in such cases information other than the fact of the heterochromia is usually scant. Thus there are questions such as: Have the full complement of iris stromal melanocytes formed? And if so, did they first form pigment and then lose it or did they never form it? These questions also are susceptible to experimental study.

INNERVATION OF IRIS AND CHOROIDAL MELANOCYTES IN THE ADULT

Because of remarkable, recent advances in neuroanatomical technique, it has become possible within the last decade to characterize terminal qualities of postganglionic autonomic nerves.[19]. Within a short span of time not only the histochemical methods listed in this report, but also electron-microscopic methods have been applied to tissues containing melanocytes and melanophores, the unusual cells which have within their cytoplasm melanin-containing granules or melanosomes.[20]

Even a cursory review of the nature of melanin-containing cells reveals their diversity: thus melanin-containing cells with stationary melanosomes are called melanocytes. And the star-shaped, melanin-containing cells in which melanosomes are reversibly translocated, first by migrating outward along the processes toward the periphery and then by migrating inward toward the nucleus, both in response to appropriate stimuli, are called melanophores. Furthermore, some melanophores are directly innervated, such as those of fish dermis;[21] while others, for instance those in amphibian dermis, have no nerves at all and are responsive to circulating neurohormones.

Melanocytes are the typical melanin-containing cells of mammals. And in a mammal the most common melanocyte is that of the epidermis. Such cells do not have a reversible cytoplasmic migration of melanosomes; however, it is not precise to say that their melanin granules are perfectly stationary, at least in a narrow sense. For melanosomes can be secreted by epidermal melanocytes into neighboring Malphigian cells. Such a process underlies the response to sunlight, which is universally called tanning. The melanocytes of the choroid and of the iris stroma share some characteristics with other melanin-containing cells, but at the same time are distinct: they contain fixed melanosomes and thus fit the usual definition of melanocyte, yet they differ from epidermal melanocytes in that

they are not known to secrete melanosomes into neighboring cells. Most important in the present context, there is increasing evidence that they are innervated.

So far as the iris is concerned this statement has been amply confirmed. There are now reports by histofluorometric observation of adrenergic nerve fibers in immediate relationship to iris stromal melanocytes in rat, rabbit, cat, and in several primate species; among these, the baboon is notable in the high density of innervation which is found in the anterior border layer of its iris.[12,22] In addition, there is light-microscopic evidence that postganglionic cholinergic nerve fibers also innervate iris stromal melanocytes.[12] Again, this observation has been made in common laboratory animals such as rabbit, cat, and monkey.

Ultrastructural investigation of the relationship of nerve to melanocyte has been scant. Nevertheless, the study by Ehinger and Falck[23] in the rat seems to be convincing. In their work on the rat iris, Ehinger and Falck were able to establish reasonable criteria by which adrenergic terminals could be distinguished from cholinergic terminals at the ultrastructural level. This was accomplished by pre-treating the rat with 5-hydroxydopamine, a compound which had previously been demonstrated to be taken up solely by adrenergic nerves, and which enhances the appearance of the 400 angstrom size, dense-cored vesicles which are considered a characteristic electron-microscopic finding of the adrenergic nerve terminal region. In contrast, similarly-sized vesicles without dense cores characterize cholinergic terminals. Using these criteria for distinguishing adrenergic from cholinergic terminals, Ehinger and Falck[23] found fine, unmyelinated nerve fibers with dense-cored vesicles, and other fine nerve fibers with vesicles lacking dense cores, both to be in frequent and close approximation to iris stromal melanocytes.

Thus for the iris stromal melanocyte the electron-microscopic and the light microscopic evidence both point strongly to a dual autonomic innervation.

It is unfortunate that Ehinger and Falck did not extend their study to the choroid, for here our knowledge of the innervation of melanocytes is far more limited. There is a tantalizing early report by Feeney and Hogan[24] in which an unmyelinated choroidal nerve was seen in close relationship to a choroidal melanocyte. However, the work was done before specific electron-microscopic criteria for distinguishing adrenergic from cholinergic terminal fields were available. Furthermore, Hogan, Alvarado, and Weddell[25] added the observation some years later that nerves are often seen close to choroidal melanocytes. Thus, enough information is available to raise a very high index of suspicion. However,

there is not enough information available for anyone to be certain about the matter. It would be most valuable if this relationship of nerve to melanocyte in the choroid could be clarified by a combined study; a study in which surgical denervation, either adrenergic or cholinergic, was done in conjunction with electron-microscopic observaton. So far as light microscopy is concerned, there is also some reason from the work of Wolter and others[28] to believe that choroidal melanocytes are innervated by autonomic nerves.[26]

At the light-microscopic level there are severe limitations to the application of histochemical techniques to the study of melanocyte innervation. These limitations derive in large part not only from the special nature of the choroid, but also from special technical problems not encountered elsewhere. As is well known, the choroid is chiefly made up of blood vessels. In addition, there are numerous melanocytes, some of which have a special relationship to the vessels and some of which are independent of blood vessels. However, virtually everywhere melanocyte and blood vessel are close one to the other. Hence, one difficulty at the light-microscopic level (when either histochemical method is used — the histofluorometric method of catecholamines or the cholinesterase technique) is that a nerve fiber once recognized cannot be arbitrarily assigned to either a neighboring blood vessel or to a melanocyte. The nature of the terminal autonomic network is such that these fibers do not have a specific ending. Rather, they are more in the nature of a fish net, arborizing widely with branches going off at different angles. Hence, in the nerve terminal region the same fiber passes by a melanocyte at one moment and then a few microns away, perhaps in the next tissue section, might drape itself about a blood vessel.

RELATIONSHIP OF ADRENERGIC INNERVATION TO THE DEVELOPMENT OF MELANIN IN THE OUTER LAYER OF THE OPTIC CUP

The adrenergic innervation appears to play no role in the developement of melanin granules within pigment epithelial cells of the retina, the pigmented epithelial cells of the ciliary body, or the pigmented epithelial cells of the iris. In humans and in monkeys these cells develop their pigment in large part before any adrenergic innervation, other than rudimentary smooth, nerve fibers in the primitive ciliary body, is present. In rats some degree of adrenergic innervation is present in the iris and ciliary body at the same time that the pigment epithelium still is actively producing melanosomes. However, just as in the monkey, there is no apparent anatomical relationship between adrenergic nerves in the uvea and the developing pigment epithelium of the retina.

In this context it is of interest that at a somewhat later time both in monkey and in rat the basal adrenergic plexus of the ciliary body is the first area of the adrenergic innervation to have a mature form. It is also noteworthy that the adrenergic innervation does in the end have a close relationship to certain pigmented epithelial cells. Thus, as shown in Figure 6A, there are adrenergic terminals in immediate apposition to the pigmented epithelial cells of the pars plana of the ciliary body of the fetal macaque monkey. And further, the adrenergic innervation to the iris dilator region, when it develops, is in close contact with the anterior epithelial cells of the iris, now having dual specialization as smooth muscle cells as well as pigment cells.

The intense fluorescence found at some time during development in all cells derived from the outer layer of the optic cup — the pigment epithelium of the retina, the pigmented epithelium of the ciliary body, and the anterior pigment epithelium of the iris — is in essence a histochemical mirror of the biosynthesis of melanin.[27,28,29] The evidence from observation is substantial: fluoresence occurs just before and just during the limited time in which melanin granules appear in these cells; fluorescence disappears completely after melanin synthesis is complete; and in the rat eye at least, a kind of visual stoichiometry is possible — in successive days the intensity of cellular fluorescence decreases just as the number of melanin granules increases. Furthermore, the emission characteristic of these cells is typical of l-dopa and, in fact, l-dopa has been extracted and measured fluorometrically from such tissues.[29]

In a very recent paper, still further information has been developed by Endo and Hu[30] on the nature of the outer layer of the optic cup. They noted a consistent absence of developing melanosomes (Stage I and II) in the posterior epithelial layer of the iris while the same developing structures were present in the early stages in the anterior pigmented epithelium. From this they deduced that the melanosomes arrive in the posterior layer ready-made from the anterior layer.

As was shown in a study by Toda and Fitzpatrick[31] on the formation of melanosomes in the embryonic chick retinal pigment epithelium, there is a stunning concordance between the histochemical observations just cited and the activity level of tyrosinase in the developing eye. In both cases there is a distinct burst of activity after which a rapid decline is experienced. There is one divergence, however. By the histofluorometric technique there comes a time when there is no activity whatsoever, while by tyrosinase assay there is always some activity left, however minimal it may be. The latter observation is important when the work of Spitznas[31] is considered. In a careful electron microscopic study of retinal pigment

epithelium in adult human, Spitznas observed a small but definite occurrence of developing melanosomes, thus indicating that some degree of melanin production takes place even in maturity.

Interestingly, l-dopa based fluorescence is also present in albinotic animals[29] (See Figure 12B). And this brilliant l-dopa fluorescence says something substantial about the nature of albinism in these albinotic species; for it certainly means that these animals have a way in which to transport tyrosine into cells, and that they have a way in which to hydroxylate it, and a way to protect l-dopa from rapid metabolic breakdown. Since it has been repeatedly shown that tyrosinase levels are very low to non-existent in albinotic animals, either another route by which l-dopa can be formed from tyrosine exists or there is a differential capacity of tyrosinase to act; that is, a specific and partial enzyme fault in which a tyrosinase retains its ability to hydroxylate tyrosine, but at the same time either cannot oxidize the resulting l-dopa to the corresponding dopaquinone or the oxidation products cannot be properly handled. The fact that in the albino guinea-pig, as reported by Winckler and Turner,[28] fluorescence persists to a later time in albino pigment epithelium than in that of pigmented species might be evidence for such a block in the metabolism of l-dopa in these animals. Alternatively, it could simply be a backward measure of the law of mass action, the evidence of a logjam later in the biosynthetic pathway.

The presence in pigment epithelial cells of widespread cytoplasmic fluorescence during the development of the melanin granules is also worth noting, for it clearly means that l-dopa is confined neither to developing melanosomes nor to a small region of the cytoplasm near the Golgi apparatus. In contrast, it is present throughout the cell (most likely in a bound form to protect it against degradation by monoamine oxidase). It needs also to be stated, however, that this observation does not lead to any conclusions about the site of production of this substance. If l-dopa were produced exclusively in one or another site in a cell, it could equally well diffuse throughout the cell, so long as it was not held in some specific way in a given region. Thus, the general distribution of this substance does little to clarify current questions about the site of tyrosinase activity within cells.[31,33,34] It does tell something, however, about the way in which it is handled after being made.

The fact that the fluorescence is widely dispersed within the cell, yet at the same time sharply delimited to individual cells is also remarkable. The absence of any discernible l-dopa fluorescence in immediately adjacent tissues is strong evidence for the existence during this early period of a restrictive function of the outer cell membrane of pigment epithelial

cells. Whether this selective permeability barrier in a newborn cell is thought of as the reverse coin of the blood-brain barrier, a sort of brain-blood barrier; or as a special aspect of amino acid transport, it is an interesting phenomenon. Certainly it offers intriguing possibilities for further experiments.

ASSAYS OF TYROSINASE ACTIVITY

In the decade since its development, the assay of Pomerantz[7] for the measurement of tyrosinase activity has gained widespread acceptance. This acceptance follows the recognition of the importance of the reaction measured and the satisfactory nature of the methodology. In the synthesis of melanin from tyrosine, the rate-limiting step is generally considered to be the first, the hydroxylation of tyrosine to form l-dopa. Thus, under normal conditions, the measurement of the rate of oxidation of tyrosine affords some measure of the rate of melanin production. This oxidation is accompanied by the formation of water. The essence of the method of Pomerantz for tyrosinase and the closely related method for tyrosine hydroxylase of Nagatsu and co-workers[35] is the measurement of this water. Both methods rely on the use of l-tyrosine tritiated in the 3,5 positions as substrate and the subsequent separation of tritiated water from residual substrate.

It might justly be asked whether the method of Pomerantz[7] differs enough from that of Nagatsu to discriminate tyrosinase from tyrosine hydroxylase. Since tyrosine hydroxylase is responsible for the conversion of tyrosine to l-dopa in peripheral adrenergic nerves (also in catecholamine-containing cells in the adrenal medulla and in the brain), might not the tyrosine hydroxylase of the rich adrenergic network to the iris also be influencing the assay? The answer appears to be "no". In the first instance there is some difference between the two assays: Tyrosine hydroxylase activity requires pteridine cofactors, cofactors which are normally added in the Nagatsu procedure and which were not added in the present work. It is also evident that the albino rabbit had very little, if any tyrosinase activity while albino rabbits and, as shown in Figure 12B, albino rats have substantial adrenergic innervation and thus also have tyrosine hydroxylase activity.

As a further check on the possibility of confusion between the two methods, selected accessory experiments were done in which diethyldithiocarbamate, a substance known to inhibit tyrosinase, was added to the pre-incubation solution. When this was done at a concentration expected to inhibit a high percentage of tyrosinase activity, just this result was obtained, adding further evidence that the assay used indeed measured tyrosinase activity.

TYROSINASE RESULTS

Once it is accepted that the assays yield a valid measure of tyrosinase activity, the question then becomes: What do they tell us about the relationship of adrenergic innervation to ocular melanin metabolism? The results as expressed in Tables I to VI indicate that a substantial relationship does exist.

So far as iris tyrosinase is concerned, interruption of the sympathetic pathways to the eye leads consistently to decline of enzymatic activity. This decline is characteristically precipitous, occurring within twenty-four hours. It is also long-lasting. Actually, there seems to be little change over time; once a new equilibrium level has been established tyrosinase activity is stable. This is certainly different from what Burn and Robinson[36] have reported to occur with amine oxidase levels. In their study it was found after removal of the superior cervical ganglion in the cat that amine oxidase levels both in iris and in nictitating membrane fell, reaching a low point in one week. Thereafter, there was a considerable rise toward control values during the next few months.

Since the role of tyrosinase in the biosynthesis of melanin is well established, it is natural to try to tie in its decline in activity with the development of heterochromia of the iris. The simplest explanation seems the most reasonable — diminished levels of tyrosinase enzyme lead to lesser production of melanin. And hence there is a failure of mainteneance — the pool of melanin within the iris stromal melanocytes is not replenished.

However, in speaking of replenishment, some form of loss or of breakdown of melanin is implied. It follows then that in the iris stroma melanin granules must either be secreted from individual melanocytes or be degraded within these cells. For the dermis such questions are easy to handle and a strong case can be made that dermal melanocytes are truly secretory cells.[37] No such case can be made for iris stromal melanocytes. Within the iris, unlike the skin, there is generally a strict demarcation of "have" and "have nots"; few, if any, cells are present in the iris stroma with intermediate numbers of melanin granules within their cytoplasm. There are iris stromal melanocytes and there are melanin-free fibroblasts.[25,38] This is not to argue that secretion of melanin granules by iris stromal melanocytes is out of the question. It only argues that there is little present evidence for such a thesis. Or, in short, if melanosomes are secreted, they are also lost from the iris.

Melanin could also be broken down within the cell. Although partial and fragmented, an increasing body of information is developing on the subject of intracellular metabolism of melanin granules. First of all,

Ohtaki and Seije[39] have demonstrated that melanosomes isolated from B-16 or Harding Passey melanomas are attacked *in vitro* by lysosomal preparations made from autologous liver; there were strong indications in this work that it was actually not the melanin itself that was being attacked but rather the protein moiety of the melanosome. In a similar vein Olson and associates[40] have emphasized that just such an attack seems to be taking place *in vivo*; they showed that the basal layer of skin contains typical lysosomal enzymes such as aryl sulphatase and acid phosphatase, localized either to individual melanosomes or in membrane bound packets of melanosomes called melanosome complexes. Olson and associates also noted a loss of characteristic morphology and markings in melanosomes within such complexes, concluding that a process of degradation was taking place. Unfortunately, if a comparable melanosome breakdown takes place in the iris stromal melanocyte, it has so far escaped detection. In fact, there is a recent paper devoted to an ultrastructural study of human iris melanosomes by Zinn and co-workers[41] in which just the opposite was found, that is, that there were no signs of degradation of melanosomes within the iris stromal melanocyte. Interestingly, in the same paper, vacuolar spaces were noted in approximately 1% of the melanosomes of the epithelial layers of the iris — vacuolar spaces that could indicate a process of breakdown. It would seem worthwhile to make further attempts to define the mechanism by which disposal of melanin granules in iris stromal melanocytes is accomplished; perhaps the most productive first step would be to search histochemically to see if hydrolytic enzymes are localized in some of the melanosomes of iris stromal melanocytes.

DENERVATION VERSUS DECENTRALIZATION: EFFECTS ON IRIS TYROSINASE.

Initially the essential similarity of the effects of decentralization and denervation on iris tyrosinase levels (Table V) is surprising. The measurements of tyrosinase activity are virtually identical, yet there certainly are differences in effects between the two procedures on the supersensitivity of smooth muscle for it has consistently been found that denervation gives a greater magnitude of supersensitivity to iris smooth muscle than does decentralization. In some test systems the difference can be five to tenfold.[42,43]

Perhaps it is fairer to compare the effect of decentralization and denervation on enzyme levels rather than on change in reactivity of smooth muscle, a change that is in good part a question of membrane properties. Although there is limited information available specifically concerning neural effects on enzymes in melanocytes,[44] a substantial body of informa-

tion is developing on the relationship of nerve function to enzyme levels within nerve itself and within some transynaptic structures. For instance, in a recent communication Black and co-workers[45] reported that two weeks after decentralization of the superior cervical ganglion in young mice, tyrosine hydroxylase, the enzyme responsible for the rate-limiting step in the production of norepinephrine and the conversion of tyrosine to l-dopa, was one-third the level found on the contralateral side. Since it has also been reported that the administration of sympathetic nerve growth factor in newborn rats elevates levels of tyrosine hydroxylase, a clear case for the inducibility of this enzyme can be made.[46] There is, in addition, evidence for a type of feedback regulation of this enzyme. In the adrenal, for instance, tyrosine hydroxylase levels decrease after systemic administration of its product l-dopa.[47]

Much valuable information is also available from studies of the effect of interruption of the sympathetic pathways to the pineal gland. In that the pineal gland in mammals can be considered representative of what in more primitive animals is the median eye, this gland holds great interest. This interest is enhanced by the fact that the product of the pineal gland, melatonin, has many relationships to pigment metabolism. Studies have been done both on the effect of sympathetic denervation and of sympathetic decentralization on the activity level of the two enzymes responsible for the conversion of serotonin to melotonin within the pineal gland. These enzymes, hydroxyindole-0-methyltransferase[48] and serotonin N-acetyltransferase,[49] both are profoundly affected when the sympathetic pathways are interrupted. There is even, in addition, some evidence that a differential effect of the two operations exists. These differences are most marked in the case of hydroxyindole-0-methlytransferase, where decentralization causes a substantially greater depletion of enzyme than does denervation.[48]

In the present study it was remarked not only that there was an adrenergic control over tyrosinase levels within iris stromal melanocytes but also that this control was exercised on a short term basis, the fall in tyrosinase being accomplished within a day. The recent work of Volkman and Heller[50] is immediately pertinent to this observation. They found that preganglionic interruption of the cervical sympathetic chain led to a noticeable effect on N-acetyltransferase levels in the pineal gland within two hours and that if instead the cervical sympathetic chain was stimulated, the activity of N'acetyltransferase rose appreciably within one hour. Thus, by analogy, it appears that ample precedent exists for short term control of enzyme levels in post-synaptic structures.

CHOROIDAL TYROSINASE

In the present studies, assay of tyrosinase from the choroid could almost be termed an afterthought; it was only after the observation in the clinical case of an apparent difference in color of the ocular fundi that attention was directed toward the back of the eye. However, once attention was focused on the choroid, several things became clear. The choroid has a high resting level of tyrosinase and the choroid undergoes the same rapid and long-lasting loss of tyrosinase activity as does the iris once the sympathetic pathway to the eye is interrupted.

The difference in tyrosinase activity in the iris and choroid is substantial. For instance, in Assay Series 6, at equal incubation times, 5 to 20 times more substrate was consumed by the choroid than by the iris on a per unit weight basis. This is really just a rough index of the difference in activity between the two. On the one hand, the difference could be greater than the estimate implies since the consumption of substrate was sufficient — some 40% to 60% — in the choroid assays to raise questions of relative availability of substrate. On the other hand, when a comparison is made of tyrosinase activity of choroid to iris, it must also be kept in mind that this is on a total weight basis, a basis that includes all components of the tissues. Perhaps a somewhat different estimate would follow extraction of melanin from both and subsequent calculation of activity on a per-unit weight of melanin basis. Simple inspection of the two tissues supplies some justification for such a suggestion. In specimens obtained for assay the choroid is jet black while the iris is usually brown.

The case for adrenergic influence over tyrosinase levels in the choroid is equally compelling to that in the iris (see Table VI). The same sequence occurs, the fall in tyrosinase activity is rapid, and thereafter tyrosinase activity settles to a new level. A careful inspection of Figure 22 shows a slight apparent downward drift in this level; the individual readings, however, are too few and too scattered to make them statistically significant. To define the matter with greater precision it would probably be better to concentration on the two extremes, for instance, to run a larger number of experiments of the one and of the 90 day duration.

CLINICAL CONSIDERATIONS

From a clinical viewpoint the first question is one of relevance. Does the sympathetic innervation have a meaningful relationship to iris color? Multiple observations in both humans[1,2,3,12] and in experimental animals[1,2,3,12,50] answer affirmatively. In children, it is generally accepted that interruption of the sympathetic pathway to the eye leads to iris

heterochromia; in adults there is no uniformity of opinion.[52,53] Since the iris heterochromia in adults is often of slight degree and is slow to develop, there are ample reasons for differences among observers. Much depends on awareness; the writer admits to seeing no cases in adults in ten years and then, once aware of the condition, to seeing three "easy" cases in a year. It should be added that the less the pigment content of the iris, the harder the diagnosis is to make; in fact in many blue-eyed patients it is just not possible to do so.

Difficult or not, the diagnosis of iris heterochromia in the adult is important. Barring the rare case with yet a second reason for iris heterochromia, iris heterochromia is a sure sign that the Horner's syndrome is not acute. With the knowledge that the condition is not of recent origin, the clinician is often then put in the position to spare the patient the risk and expense of unnecessary diagnostic procedures.

Although the present report is devoted to a demonstration of neurohumoral or neurohormonal control over tyrosinase activity, this enzyme might also be sensitive to other agencies, agencies of a neural type such as acetylcholine or agencies which are entirely outside of normal physiology. Control over enzyme activity can be exerted thus in a variety of ways. Just such a variety of types of heterochromia presents themselves to the ophthalmologist. Thus, it would be of immediate interest to ascertain what role, if any, tyrosinase plays in the development of several types of iris heterochromia as they present clinically. For instance, does tyrosinase play a role in the iris discoloration which occurs in some instances of retained intraocular metallic foreign body? True, there is generally tissue staining from the metal ion, but that does not eliminate tyrosinase as a participant. Such considerations are most appropriate in the case of copper. Copper is a necessary part of the tyrosinase enzyme and substances which bind copper also inhibit tyrosinase activity; what is then the effect of a surfeit of copper? Or, perhaps, more important, what effect does inflammation have on tyrosinase activity? Here, it would be of special interest to know if a long-lasting, low-grade inflammation such as the clinician witnesses in Fuchs' heterochromic cyclitis were also accompanied by a long-lasting depression of tyrosinase activity.

THE CHOROID AND TYROSINASE ACTIVITY

The finding in a single patient of a modest difference in choroidal pigmentation, a difference which might well have preceded the onset of Horner's syndrome, can only be ascribed a limited significance in and of itself. However, when the laboratory information about sympathetic con-

trol of choroidal tyrosinase levels is added to the clinical observation, there is enough to warrant further investigation to find if indeed choroidal heterochromia is or is not a part of a long-lasting Horner's syndrome. If it is, credit certainly is owing to Angelucci[2] who some eighty years ago described choroidal changes in experimental animals after interruption of the sympathetic pathways.

For the choroid, the general finding that there remains in the adult animal a substantial level of tyrosinase also merits further attention. There are scattered reports of changes of choroidal pigmentation under light stimulus[54] and of seasonal changes in light sensitivity,[55] both of which are at present without full explanation. A decreased scotopic sensitivity during the summer months is likely due to a slight degree of light damage to photoreceptors, such as has been noted in those exposed on bright, sunny beaches.[56] However, there is no sufficient explanation at present for the observation of Busacca[54] of light-induced modification of choroidal pigmentation in the fundus. Nor is there sufficent information available about the turnover rate of melanin in choroidal melanocytes to explain why so high a level of the biosynthetic enzyme, tyrosinase, is present in these cells in the first place.

SUMMARY

Iris stromal melanocytes receive both adrenergic and cholinergic innervation. It is probable, but not certain, that choroidal melanocytes are similarly innervated.

Tyrosinase, the rate-limiting enzyme in the biosynthesis of melanin, is present both in the iris and in the choroid. Interruption of the sympathetic pathways leads to a rapid decline in the tyrosinase activity in both choroid and iris. The magnitude of the decline of tyrosinase levels was the same in animals in which the superior cervical ganglion had been removed and in animals in which there had been a preganglionic interruption of the sympathetic pathways; in neither case did tyrosinase return to normal in the ensuing months.

During development there is a reciprocal relationship between the fluorescence intensity of l-dopa and the accumulation of melanin granules in the pigmented epithelial cells derived from the outer layer of the optic cup.

In the developing eye, melanin granules form in the pigment epithelium before the adrenergic terminal network is established. In contrast, some evidence of adrenergic innervation is always present during the

development of stromal melanocytes in iris and choroid; however, there is little evidence of direct contact and the innervational network is immature during the major part of stromal melanocyte development.

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