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# THE AFFINITY OF MELANIN FOR CHLOROQUINE*

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The localization of chloroquine in areas of melanin pigmentation has recently been established. Zvaifler and his co-workers (1) found that the concentration of this drug in the eyes of pigmented rats paralleled the degree of melanin pigmentation. In addition, they noted that chloroquine persists at high levels in the iris and choroid for at least 28 days whereas the concentration decreases rapidly in the other tissues examined (2). However, in the studies of Zvaifler *et al.*, no information was obtained for skin pigment. A study of the possible affinity of chloroquine for skin melanin became feasible with the acquisition of a strain of pigmented hairless mice which develop melanotic plaques at sites of repeated ultraviolet irradiation (3).

## METHODS

The experimental animals consisted of 40 pigmented and 40 albino 2½ month old, random-bred, female hairless mice. They were housed in metal cages and fed on unrestricted quantities of Wayne Laboratory Blox and water. The ultraviolet source was an Hanovia air-cooled, high-pressure mercury contact lamp. This lamp emits $2.12 \times 10^5$ ergs/cm$^2$/sec. of mid-ultraviolet energy (2800–3200 Å) at a distance of 3.4 cm. Measurements of the energy output were made with an Hanovia ultraviolet meter (Model ZV-971).

Each mouse received a 5 second exposure of ultraviolet to the posterior third of the back three times a week. By 10 weeks the pigmented mice had developed deeply melanotic plaques, and the albino animals developed erythematous non-melanotic plaques at the irradiated sites (Fig. 1). The ultraviolet radiation was discontinued at this time, and each of the animals was given a single intraperitoneal injection of 0.5 mg chloroquine diphosphate (equal to 0.4 mg chloroquine base). Equal numbers of the albino and pigmented animals were sacrificed at 24, 48, and 72 hours, and then weekly for three weeks. Specimens of skin for histologic studies were obtained and the irradiated areas were excised from the remainder of the skin. An additional albino and pigmented animal was sacrificed at each time period for determination of the dry weight of the tissue, since most of the specimens were too small to afford an aliquot for dry weight calculations. Dry weighing was performed by drying the skin in a vacuum oven at 90° C for 24 hours. The assay procedure followed was modified from that described by Brodie *et al.* (4).

Each skin specimen was weighed, cut into fine pieces with scissors, and ground at 10° C with 12 ml 0.1 N HCl for 2 minutes in a Servall Omni-Mixer. The material was then transferred to a 50 ml glass-stoppered centrifuge tube containing 9 ml 0.2 N NaOH and 10 ml heptane. The tubes were shaken vigorously for 30 minutes, centrifuged at 5,000 × *g* for 15 minutes, and the heptane phase containing the chloroquine removed. The heptane was washed × 2 with 0.1 N NaOH and finally extracted into 2 ml 0.1 N HCl. Three drops of ethanol were added at each step to decrease adsorption of chloroquine to the glass of the tubes. For fluorometry, 1.6 ml of the 0.1 N HCl containing the chloroquine was transferred to a fluorometer tube containing 0.33 ml 0.5 N NaOH and 0.5 ml boric acid buffer, (5 vols 0.6 M boric acid in 0.6 M KCl added to 3.2 vols 0.6 N NaOH). To each tube was added 0.16 ml of neutral 1.0 M *l*-cysteine HCl to prevent oxidation of the chloroquine during irradiation. After 30 minutes, to allow for reaction between cysteine and oxygen, the tubes were irradiated for 1 hour with ultraviolet light from a Burdick high pressure mercury lamp type QA-450-N at a distance of 18 inches. The amount of fluorescence which developed was read in a Turner Fluorometer (Model 111), using a Corning 7-60 as the primary filter (passes light below 365 mμ) and a 110-816 (2A) as the secondary filter (passes light above 415 mμ). The instrument was zeroed with a blank and readings were compared with standard solutions of chloroquine handled in exactly the same manner as the unknowns. In addition, the skin of animals which had not been injected with chloroquine was assayed to determine the amount of interfering fluorescence in skin itself. This value was found to be consistently very low but constantly present and was subtracted from the actual determinations of chloroquine levels.

## RESULTS

Histologic examination revealed hyperkeratosis, hypergranulosis, acanthosis, and a thick-

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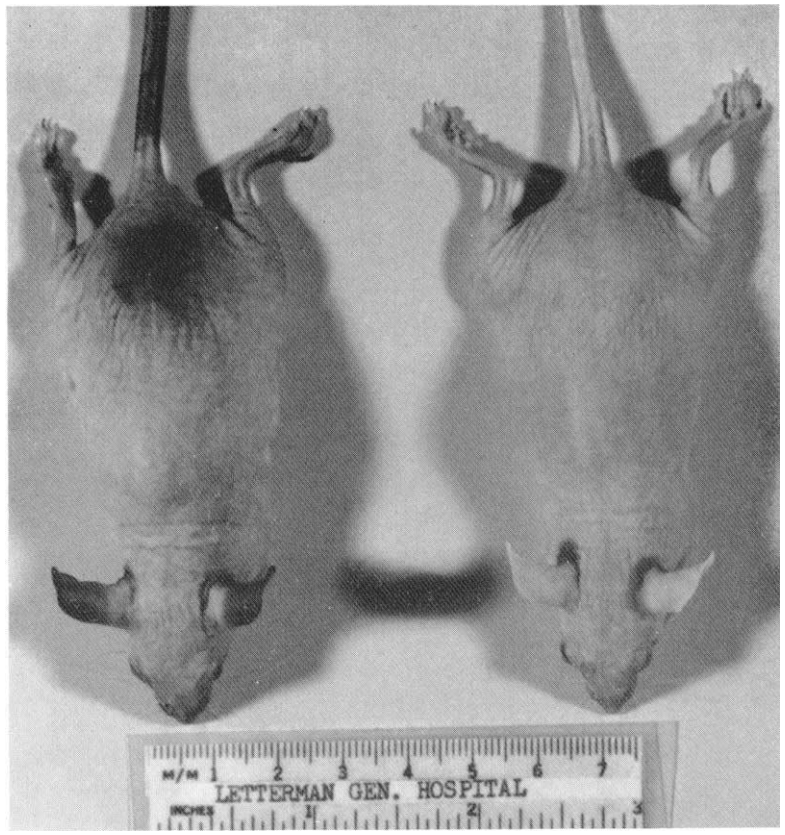


FIG. 1. An albino hairless mouse on the right and a pigmented mouse on the left. Both animals have received 10 weeks of ultraviolet light to the posterior third of the back. The melanotic plaque is evident in the pigmented mouse.

ened dermis, with an increased number of mast cells, fibrocytes, and inflammatory cells in both the amelanotic and melanotic plaques (Figs. 2 and 3). The tissues of the pigmented mice differed from that of the albino mice only in the dense accumulation of dermal melanocytes and melanin in the pigmented areas. (The pigment stained positively for melanin with the Fontana stain). The non-irradiated skin of these animals showed an epidermis of 2–3 cell layers and a thin dermis with degenerating hair follicles, epithelial cysts, and some mast cells, (Figs. 4 and 5). In contrast to the normal skin of the albino, the normal skin of the pigmented mice contained a few scattered dermal melanocytes.

The results of the chloroquine assays are presented in Table I. In addition, the differences between the chloroquine concentrations in the irradiated and non-irradiated skin are presented graphically in Figure 6.

Twenty-four hours after the single injection, similar amounts of chloroquine were found in the skin of the albino and pigmented mice though the irradiated areas showed much higher values in both types of animals. By 48 hours the concentration in the non-irradiated skin of both the albino and pigmented mice had fallen to undetectable levels. The amount of chloroquine present in the irradiated albino tissue was also reduced, but significant levels were still present at 72 hours. By 7 days no chloroquine was detectable in any part of the albino skin. In contrast, the concentration of the drug in the irradiated plaques of the pigmented mice increased over the first 2 days, reaching its peak at 48 hours. Though some reduction of the chloroquine concentration in these pigmented tissues occurred thereafter, significant levels persisted for at least three weeks. Pigmented skin has not yet been examined for its chloroquine content after more than three weeks subsequent to administration of the drug.

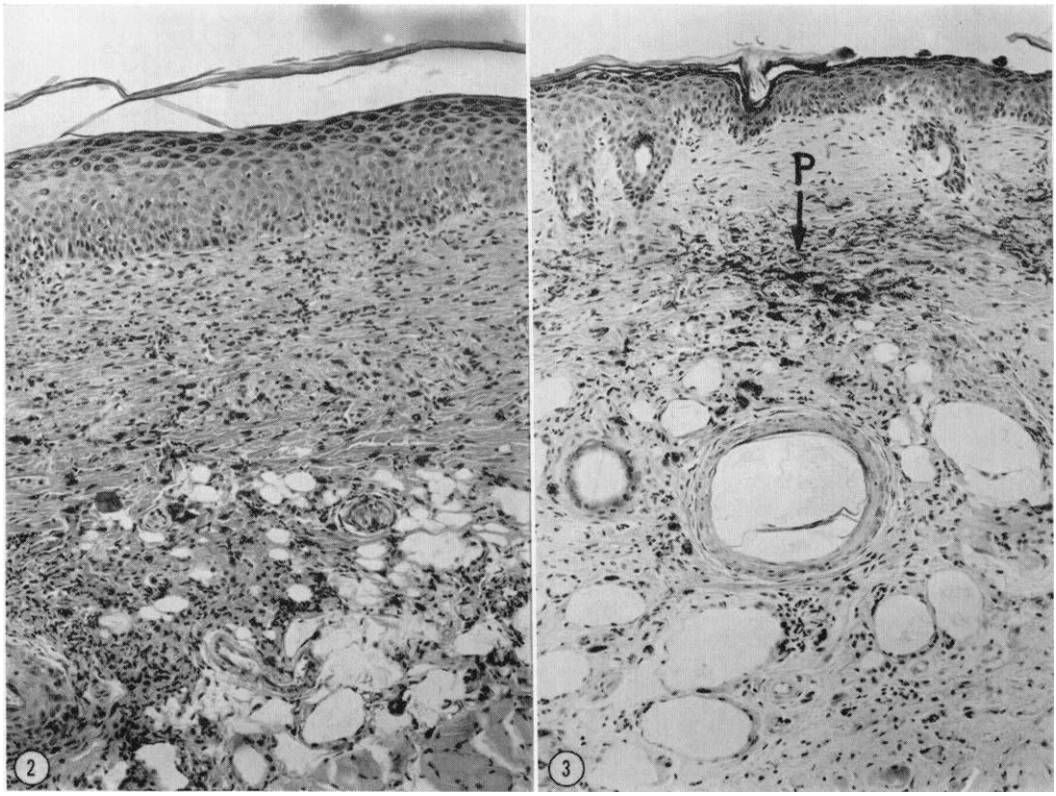


FIG. 2. Albino plaque showing hyperkeratosis, hypergranulosis, acanthosis and thickening of the dermis with accumulation of fibrocytes and mast cells.

FIG. 3. Pigmented plaque showing hyperkeratosis, hypergranulosis, acanthosis and thickening of the dermis with accumulation of fibrocytes and mast cells. A dense accumulation of melanin and melanocytes is present in the mid dermis (p). The acanthosis present in the amelanotic plaque, (Fig. 2), is more striking than that seen in the melanotic plaque, (Fig. 3). This difference was neither a consistent nor a significant variation in the response of these two skin types to radiation.

## DISCUSSION

Clinical observations have established that pigmentary abnormalities occur in a significant number of patients receiving chloroquine therapy (5–7). These changes consist primarily of loss of hair color and formation of blue-black pigmented patches involving pretibial, facial, and palatal areas. Histologically, this skin and mucosal discoloration apparently is due to the deposition of clumped yellow to dark brown pigment in the dermis. Recent studies suggest that similar alterations in the ocular pigment system may be related to the irreversible retinal damage that has been reported following long term chloroquine administration (8–10).

The mechanism or mechanisms responsible for the pigmentary aberrations are not clear. The results of our study as well as those reported by Zvaifler *et al.* (1, 2) indicate that chloroquine not only has a remarkable affinity for cutaneous and ocular melanin, but that it is not readily released from the pigmented tissues. Thus, this drug most likely has a direct effect on the pigment systems. The *in vitro* studies of Perez *et al.* (11) suggest that chloroquine binding to melanin may be mediated through a charge transfer process. *In vivo* data, however, have been difficult to obtain, partly because of the limited amount of tissue available for study when the eye is used for study. The cutaneous pigmented plaque described in the present paper affords a larger pigmented model for more thorough *in vivo* evaluation of the pigmentary alterations.

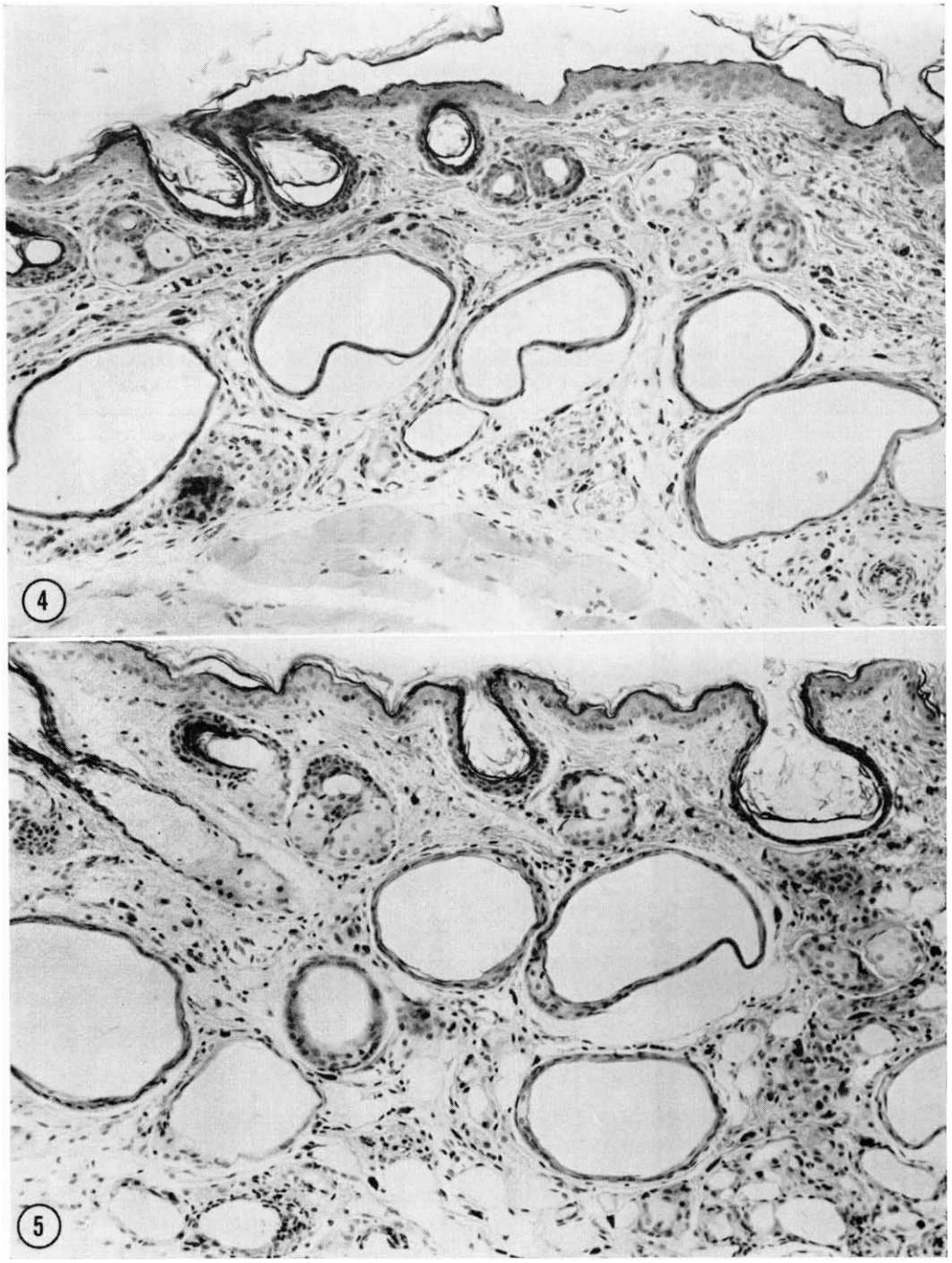


FIG. 4. Normal skin of the albino showing a 2 to 3 layer epidermis, and a thin dermis with sebaceous glands, epithelial cysts and degenerated hair follicles.
FIG. 5. Normal skin of the pigmented mouse.

TABLE I

*Comparison of the chloroquine concentration of the irradiated and non-irradiated skin of pigmented and albino mice following intra-peritoneal injection of chloroquine diphosphate*

| Time after Injection | PIGMENTED | | | | ALBINO | | | |
|---|---|---|---|---|---|---|---|---|
| | Irradiated | Non-irradiated | Difference | Number of Animals | Irradiated | Non-irradiated | Difference | Number of Animals |
| 24 hours | 2.80 | 0.67 | 2.18 | 6 | 3.90 | 0.92 | 2.98 | 4 |
| 48 hours | 3.07 | 0 | 3.07 | 4 | 1.16 | 0 | 1.16 | 3 |
| 72 hours | 1.94 | 0 | 1.94 | 2 | 1.09 | 0 | 1.09 | 2 |
| 1 week | 1.54 | 0 | 1.54 | 2 | 0 | 0 | 0 | 4 |
| 2 weeks | 2.63 | 0 | 2.63 | 2 | 0 | 0 | 0 | 4 |
| 3 weeks | 1.07 | 0 | 1.07 | 6 | 0 | 0 | 0 | 6 |

The values given are averages of the total number of animals in each group, expressed as micrograms of chloroquine per gram dry weight of skin.

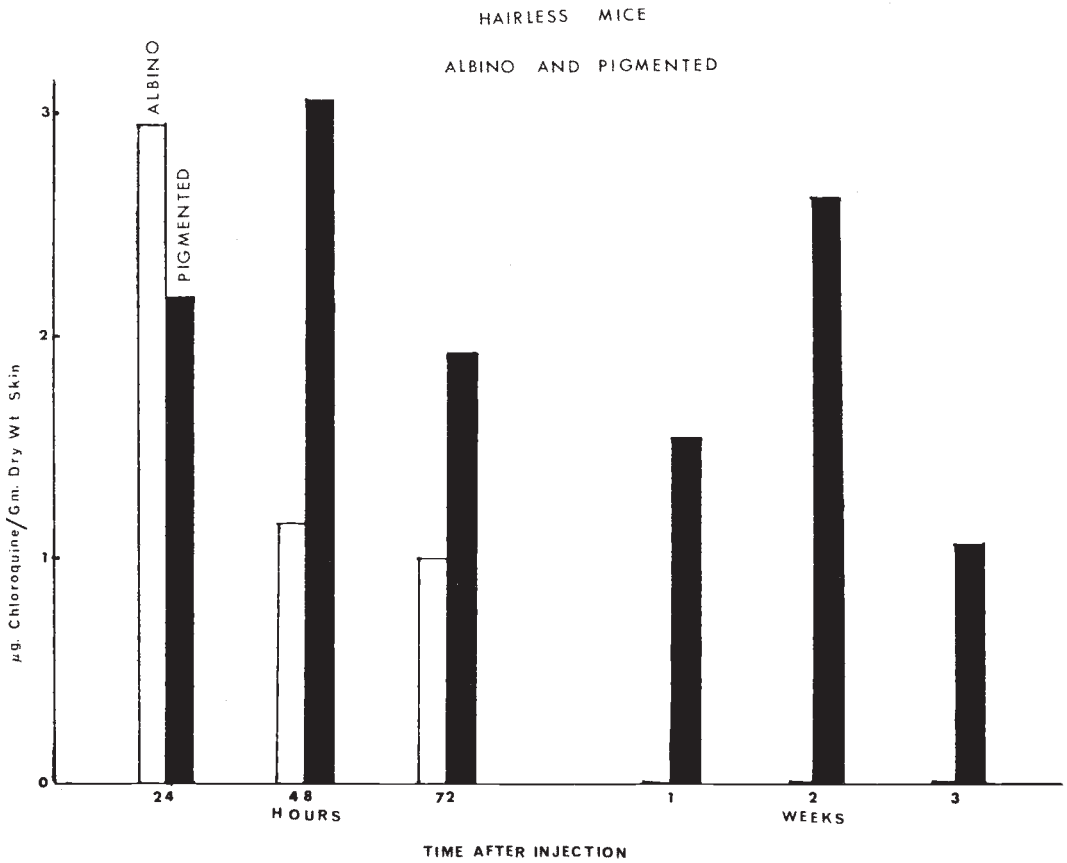


FIG. 6. Graphic representation of the difference in the chloroquine levels in the irradiated and non-irradiated skin of albino and pigmented mice.

SUMMARY

By the use of pigmented hairless mice which developed a darkly melanotic plaque at the site of ultraviolet irradiation, it has been possible to demonstrate the affinity of dermal melanin for chloroquine. The chloroquine levels in the albino control mice, which developed non-melanotic plaques at the irradiated sites, fell rapidly to zero within one week after a single intraperitoneal injection of the drug. The melanotic plaques of the pigmented mice, on the other hand, contained high levels of chloroquine for at least three weeks. Thus, the hairless mouse provides a model for studying the binding of drugs to melanin.

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## DISCUSSION

Dr. Thomas B. Fitzpatrick, Boston, Mass.: These two papers illustrate what we have known for a long time from enzymic and electronmicroscopic studies of the melanin in the retinal pigment epithelium. Melanin formation begins in the retinal pigment epithelium before it occurs in the melanin-forming cells of any other part of the melanocyte system. This is beautifully illustrated in the chick embryo which has very large eyes that darken early in the course of development, *i.e.*, within the first few days. In the chick, melanin is formed in the retinal pigment epithelium before it is formed in other parts of the melanocyte system; melanin formation also ceases in this tissue earlier than in other parts of the system. Enzymic studies provide no evidence that melanin formation continues in the retinal pigment epithelium of the chick after the 18th day of development. In man, events follow a similar course. This would explain the relatively slow turnover of chloroquine since the melanosomes and melanin granules of the retinal pigment epithelium are not ejected from melanocytes into Malpighian cells as they are in the epidermis and the hair bulb.

Dr. M. A. Pathak, Boston, Mass.: I would like to know why the authors did not inject chloroquine in the first ten-week period when the mice were being irradiated. I feel that the binding or incorporation of chloroquine in to melanin would be more during the first ten-week period. At this time the process of melanogenesis (new melanin formation) would be progressing much more rapidly. During this interval the synthesis of premelanosomes, melanosomes and melanin granules would be greatly augmented and hence the incorporation or binding of chloroquine during melanogenesis would be more likely to be augmented.

Dr. W. Mitchell Sams, Jr. (in closing): I would like to thank the discussors for their comments. In answer to Dr. Fitzpatrick's question about rates of turnover, the turnover rate of chloroquine is not necessarily related to the turnover rate of melanin. This is because the binding of chloroquine to melanin is most likely by a charge transfer process since chloroquine is a fair electron donor and melanin is a rather good acceptor (Szent-Györgyi, A., An Introduction to Submolecular Biology and Perez *et al*, Arthr. and Rheu. *7:* 337, 1964). This bond is probably not very strong, not nearly as strong

as with chlorpromazine (which is a superb electron donor), and thus the chloroquine might be "washed off" the melanin molecule long before the latter turned over. In reply to Dr. Pathak, ultraviolet irradiation of the animals was stopped prior to injection of chloroquine. Dr. Stoughton asked if this binding phenomenon could be related to the amount of melanin in the retina and macular degeneration of the latter. The answer, at this point, is that this seems likely but is not proven. It is well known, however, that chloroquine can be found in much higher concentration in the pigmented iris and choroid than in the non-pigmented cornea, retina, and sclera. (Zvaifler *et al*, Arth. and Rheu. *5:* 667, 1962.) Some of the known individuals who have developed macular degeneration have been Negroes, who would be expected to have large amounts of ocular melanin, while others have been Caucasians. The total number, however, is not sufficient at present to make any statistical evaluation.