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THE PATHOLOGY OF
PYROPHEOPHORBIDE α
PHOTOSENSITIVITY IN
ALBINO RATS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
GEORGE C. JERSEY
1969

THESIS

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ABSTRACT

THE PATHOLOGY OF PYROPHEOPHORBIDE a PHOTOSENSITIVITY IN ALBINO RATS

by George C. Jersey

Seventy-gram albino rats of both sexes were consistently photosensitive 24 hours after 3 mg. of pyropheophorbide a was given by intravenous injection. Photosensitization was induced by exposure to light from fluorescent lamps. In addition to the usual clinical and pathologic manifestations of photosensitization, a severe chorio-retinitis was produced that led to complete destruction of the retina. Cataracts developed in 38% of the rats. Rats given only the vehicle by intravenous injection were not photosensitive and intraocular lesions did not develop when exposed to light. Rats sensitized with pyropheophorbide a but held under the usual lighting conditions of the animal room did not have lesions.

Rats fed 7 to 15 mg. of pyropheophorbide a developed the same lesions when exposed to light as those sensitized by the intravenous route. An acute dacryoadenitis was produced in both the vehicle control and treated rats that were exposed to light.

THE PATHOLOGY OF PYROPHEOPHORBIDE a
PHOTOSENSITIVITY IN ALBINO RATS

By
George C. Jersey

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INTRODUCTION

Photosensitization results from a photochemical reaction sensitized by a variety of light absorbing compounds. Hashimoto, Naito and Tsutsumi (1960) photosensitized rats, mice and cats by feeding the viscera of abalones (abalones are univalve marine mollusks). Pyropheophorbide a, a chlorophyll derivative, was identified as the sensitizing compound. Since many food substances contain chlorophyll, there is a possibility of a photosensitizing derivative being formed during the manufacture of one of the many new foods developed by food scientists. The proposed use of unusual protein sources such as algae increases this possibility.

This study was conducted in conjunction with members of the Department of Food Science in support of their research on chlorophyll and its derivatives. The objective was to characterize the response of the rat to an experimentally induced photosensitization with major emphasis on the determination and description of the histologic changes. Pyropheophorbide a which had been derived from chlorophyll by laboratory methods was used to sensitize the rats to light.

LITERATURE REVIEW

Santamaria and Prino (1964) listed 380 chemical compounds that were capable of sensitizing one or more biological systems to damaging photochemical oxidation. Certain drugs, several porphyrin derivatives and numerous dyes were the more important sensitizers from the standpoint of experimental and clinical photosensitizations of man and animals.

The mechanism of the chemical reactions involved is not completely known; however, it is generally agreed (Spikes, 1968; Santamaria and Prino, 1964) that the sensitizing molecules absorb quanta of radiant energy and become chemically activated. The activated molecules then react with substrate molecules normally present in the system. Molecular oxygen is consumed in the process (Blum *et al.*, 1935). The net reaction is an oxidation that results in damage to the host system. The molecules of the photodynamic agent are regenerated (Spikes, 1968; Santamaria and Prino, 1964).

Sunlight is the source of radiant energy for naturally occurring photosensitizations. Sunburn is also caused by the radiant energy from the sun but the mechanism of sunburn is distinctly different (Blum, 1941). Ultraviolet light of wavelength shorter than 320 nm. (Coblentz and Stair, 1934) causes sunburn. The energy of the radiation in the sunburn range is sufficient to disrupt molecular conformations by direct action (Johnson *et al.*, 1968). Intermediate substances are not

required and the damage occurs in the absence of molecular oxygen (Blum *et al.*, 1935). Ordinary window glass absorbs the ultraviolet energy in the sunburn range thus sunlight passing through a closed window does not cause sunburn. Conversely, a sheet of window glass is commonly inserted between the light source and the animal in experimental photosensitizations (Blum, 1941). The tissue response to sunburn is delayed and inflammation is not apparent for several hours (Clare, 1952). Response of the tissues in photosensitizations is immediate and inflammation may be noticeable within a few minutes after exposure begins (Blum, 1941).

Maximal absorption of light by a photodynamic agent takes place only at or near specific wavelengths. Wavelengths corresponding to the absorption maxima of a given agent most effectively excite the molecules of that compound (Blum, 1941).

The photodynamic diseases of animals were classified by Clare (1952) on the basis of the origin of the photodynamic agent or the route by which it gets into the peripheral circulation. He formulated three types, as follows:

1. Primary photosensitivity. The sensitizing agent is ingested as part of the diet and accumulates in the tissues without predisposing lesions of other organs. The anthelmintic, phenothiazine, hypericin found in plants of the genus *Hypericum*, and fagopyrin obtained from buckwheat (*Fagopyrum esculentum*) are the most common primary photosensitizers of farm animals. The photosensitizers fed or injected into animals for experimental photosensitizations are usually of this type.

2. Hepatogenous photosensitivity. The photodynamic agent accumulates in the tissues due to hepatic damage or obstruction to the flow of bile. The sensitizer in this instance is normally eliminated in the bile. Most photosensitizations of ruminants are of this type. Phylloerythrin, a normal product of chlorophyll digestion, is the only substance known to accumulate in the body by this mechanism. It is the sensitizing agent of facial eczema of sheep in New Zealand and Australia.

A congenital hepatic insufficiency has been reported in the Southdown (Jamieson and Swan, 1952; Hancock, 1950) and Corriedale (Cornelius *et al.*, 1965) breeds of sheep. Phylloerythrin is the sensitizing agent but there are no gross or microscopic lesions in the liver or biliary system. Onset of the disease occurs when the lambs begin to consume foods containing chlorophyll. Withholding the lambs from pasture during the daytime prevents onset of signs and they grow to normal maturity. Lambs allowed to graze during the daytime invariably die.

3. Photosensitivity due to abnormal porphyrin synthesis. The diseases under this heading are heritable abnormalities of porphyrin metabolism in which aberrant porphyrin compounds of endogenous origin accumulate in the body. Several variants of the disease have been identified in man (Cripps, 1967). Photosensitivity is not a constant finding in all porphyrias. Congenital porphyria has been reported in cattle more frequently than in other farm animals. Porphyrin cattle are highly sensitive to light (Wass and Hoyt, 1963).

Hashimoto *et al.* (1960) photosensitized rats, mice and cats by feeding the livers of abalones. Rabbits force-fed the livers of the abalones were not photosensitive. Hashimoto and Tsutsumi (1961)

prepared a partially purified ether extract from livers of abalones which sensitized rats when added to the diet. Tsutsumi and Hashimoto (1964) further purified the compound and identified it as pyropheophorbide a, a chlorophyll derivative.

Clare (1953) had previously photosensitized rats with pyropheophorbide a. He noted the similarity of this compound to phylloerythrin. The compound Clare (1953) studied was formed in the process of drying green millet, clover and rye grass in a coke dryer. It could not be isolated from any of these plants before drying them. Clare (1953) also found that rats were made extremely photosensitive when the feces of sheep were added to their diet. Rimington (1937) isolated phylloerythrin in rather abundant quantities from the feces of sheep that were grazing green pasture. Clare (1953) concluded that the liver of the rat is less able to eliminate the photosensitizing chlorophyll derivatives than that of herbivorous species. He did not believe the photosensitizations caused by the chlorophyll derivatives were significant except as they occur in herbivores secondary to hepatic damage.

The work of Hashimoto *et al.* (1960) was prompted by old reports of a peculiar dermatitis that occurred in Japanese fishermen while catching abalones. The dermatitis developed when the fishermen were out on the boats in bright sunlight. It was rapid in onset and had all the characteristics of a photosensitization. The disease disappeared some 50 years ago when abalone fishing was prohibited from April through October. The circumstantial evidence suggests the fishermen were being sensitized by the pyropheophorbide a found in the abalones. The fishermen were known to consume large quantities of the abalone. This was

the first report of a photosensitizer originating from a food substance of animal origin and also the first report of a dietary photosensitivity of man.

The many reports of both experimental and clinical photosensitizations contain only an occasional reference to blindness as a part of the disease. Conjunctivitis and keratitis were reported by several authors (Cornelius *et al.*, 1965; Clare, 1955; Mathews, 1938).

The lesions of the conjunctiva and cornea were presumed to be the cause of blindness when it occurred. Keratitis is the most often observed lesion in phenothiazine-sensitized photosensitizations of calves (Whitten *et al.*, 1946). Clare *et al.* (1947) specifically studied the ocular aspects of phenothiazine-induced photosensitization of calves, and lesions of the retina and choroid were not observed.

Cloud *et al.* (1960) reported damage to the eyelids, cornea, iris and lens in guinea pigs treated with methoxsalen and irradiated with long ultraviolet light. Mice treated for a longer period with the same compound had cataracts, devascularization of the iris and abnormal dilatation of the pupil (Cloud *et al.*, 1961).

Walkowicz (1956) produced a lesion of the retina by sensitizing rabbits with eosin and irradiation of the eye with a very narrow beam of light. He described the lesion as being similar in appearance to central serous retinopathy upon ophthalmoscopic examination. The histologic preparations contained areas of edema between the choroid and retina, in the nerve-fiber layer and in the layer of rod and cone cells.

Jaffe (1950) reported retinal hemorrhages in a patient suffering from acute porphyria. Barnes and Boshoff (1952) examined the eyes of

84 patients with various forms of porphyria in South Africa. Lesions of the fundus were present in 42 of the 84 patients. In comparison, fundic lesions were observed in only 311 of 7,548 nonporphyric patients. A variety of retinal and choroiditic lesions was described in the porphyric individuals.

Solar retinitis occurs typically in individuals who view solar eclipses with the unshielded eye (Newell, 1964; Duke-Elder, 1926). A number of recent reports indicate a vulnerability of the retina to damage by light. Noell *et al.* (1966) reported their extensive studies of rats exposed to intense light. Irreversible damage to the retina was produced by light applied for less than 1 hour or for up to 2 days depending upon the experimental conditions. Electroretinographic measurements were used to detect the earliest retinal changes. Histologic manifestations of retinal damage were present as early as 4 hours after exposure. However, widespread irreversible retinal damage did not occur at normal body temperature when exposure was for less than 24 hours. The retinal changes were appreciably accelerated by increasing the body temperature to 104 F. Dantzker and Gerstein (1969) studied the retinal vascular changes in rats exposed to light for 24 hours. In trypsin-digested whole-mount preparations of the retina, the density of the capillary network was markedly reduced with narrowing and fibrosis of the capillaries that remained. Additional exposure to light produced more widespread, but not more severe, vascular degeneration.

Isolated calf's retina irradiated with wavelengths greater than 320 nm. underwent an oxygen dependent enzymatic damage (Pierpaoli and Santamaria, 1961). Liver and kidney slices treated similarly were not

damaged. Santamaria and Prino (1964) suggested that the high levels of natural photodynamic substances may contribute to the sensitivity of the retina for normal vision or may partially explain the retinal damage caused by intense light.

Chloroquine, an antimalarial drug, is a known photosensitizing agent (Allison, 1967; Slater and Riley, 1966) and is associated with an increased incidence of retinal lesions in man (Crews, 1966; Carr *et al.*, 1968). Chlorpromazine, a tranquilizing drug derived from phenothiazine, is another therapeutic agent known to cause photosensitivity. It has recently been linked with retinal lesions (Crews, 1966; Mathalone, 1966; Grunby *et al.*, 1966). Crews (1966) suggested the possibility of a phototoxic mechanism in the retinal diseases associated with these drugs.

Mathews (1938) sensitized rats by feeding extracts of the leaves of lechuguilla (*Agave lecheguilla*) and by feeding buckwheat (*Fagopyrum esculentum*). The rats were photosensitized by exposure to direct sunlight. The signs and symptoms were the same regardless of the photodynamic agent used. He observed pruritis, erythema, lacrimation, edematous swelling of the face and ears, and exophthalmos. Later developments were superficial necrosis of the ears and opacity and ulceration of the cornea.

Microscopically, Mathews detected an early hydropic degeneration of the cutaneous capillary endothelium, followed by serous exudation that spread through the dermis and deeper connective tissue. Leukocytes and plasma cells infiltrated the edematous area. Extensive necrotic changes accompanied by an infiltration of neutrophils led to sloughing of the epithelium and scab formation.

MATERIALS AND METHODS

Experiment I

Twenty-four rats^{*} from Sprague-Dawley stock were used for this experiment. They averaged 70 gm. and were approximately 4 weeks of age. They were housed individually in galvanized wire cages and were provided food and water *ad libitum*. Environmental factors such as room temperature and extraneous room light were constant for all groups. An outline of the experiment is presented in Table 1.

The rats were divided into 4 groups of 6. There were equal numbers of males and females in each group. A group consisted of one vehicle control, one dark control, and 4 experimental subjects.

A 0.75% test solution of pyropheophorbide a^{**} was prepared using a mixture containing 5% desoxymethyl sulfoxide (DMSO)^{***} and 95% propylene glycol[#] as the vehicle.

The rats serving as experimental subjects and as dark controls were given 0.4 ml. of the test solution by injection into the lateral tail vein. The vehicle control rats were given 0.4 ml. of the DMSO-propylene glycol vehicle by the same route.

^{*}Spartan Research Animals, Haslett, Michigan.

^{**}Prepared by Mr. Mark Love of the Department of Food Science.

^{***}K & K Laboratories, Inc., 1211 Express St., Plainview, N.Y.

[#]Eastman Organic Chemicals, Rochester, N.Y. 14603.

Table 1. Experiment I. Experimental design for intravenous injection study of pyropheophorbide a

	Number of Rats	Sex	Dose (mg.)	Number of Exposures	Killed Post- exposure (days)	Amount of Vehicle (ml.)
<u>Group A</u>						
VC*	1	M	0	2	0.5	0.4
DC**	1	F	3	0	0.5	0.4
T***	4	2M,2F	3	2	0.5	0.4
<u>Group B</u>						
VC	1	F	0	2	3	0.4
DC	1	M	3	0	3	0.4
T	4	2M,2F	3	2	3	0.4
<u>Group C</u>						
VC	1	M	0	2	7	0.4
DC	1	F	3	0	7	0.4
T	4	2M,2F	3	2	7	0.4
<u>Group D</u>						
VC	1	F	0	2	14	0.4
DC	1	M	3	0	14	0.4
T	4	2M,2F	3	2	14	0.4

*VC = vehicle control (given vehicle and exposed to light).

**DC = dark control (sensitized with pyropheophorbide a but not exposed to light).

***T = treated rats (sensitized with pyropheophorbide a and exposed to light).

The source of light was a fixture* containing 12, 110 watt, fluorescent lamps** 48 inches in length. The exposures were conducted on 5 rats at a time so that the vehicle control rat and the 4 experimental subjects within a group were exposed simultaneously. The dark control rat was held in its cage under the normal room conditions. No attempt was made to screen out the ordinary room light.

For the exposures, the rats were placed on a sheet of natural plywood 19 inches below the bank of lights. Each rat was confined beneath an exposure chamber made from a transparent disposable mouse cage.*** Numerous holes were drilled in the sides of the mouse cage to provide adequate ventilation. The temperature beneath the lamps was approximately 30 C. Light intensity beneath the exposure chambers was measured with a photocell.# The average intensity was 18,050 luxes as measured for the 5 different positions of the exposure chambers on the plywood platform.

The rats were exposed to the light source for 2 periods of 90 minutes each. The first exposure was 24 hours after administering the pyropheophorbide a and the second exposure was after an additional 24 hours. The rats were killed with pentobarbital sodium at intervals of 12 hours (Group A), 3 days (Group B), 1 week (Group C), and 2 weeks

*Sherer-Gillett Co., Marshall, Michigan.

**Model F48T12/CW/VH0. Sylvania Lighting Products, Danvers, Mass.

***Lab-Line Laboratory Cages, Inc., Melrose Park, Ill. 60160.

#Intensity Measurement Photocell, Model 603. Neston Instruments, Newark, N.J.

(Group D) following the second exposure. The rats were necropsied immediately after death. Tissue specimens were collected from all organs and fixed in either 10% acetate buffered neutral formalin or in Zenker's fluid.

Special attention was given to the eyes. Each eye was carefully dissected from the orbit along with as much orbital tissue as possible and the entire mass was fixed and processed without further dissection. One eye from each rat was fixed in Zenker's fluid and the other in formalin.

Paraffin sections were cut at 6 microns from both eyes and both ears. Only the formalin fixed tissues from other organs were examined histologically.

The following staining procedures (Armed Forces Institute of Pathology, 1968) were used: hematoxylin and eosin, Gomori's method for iron, Kossa's method for calcium, toluidine blue for mast cell granules, and alizarin red for calcium.

In addition to the experiment outlined above, 2 smaller experiments were conducted. Only those aspects of these experiments which differed from Experiment I will be described.

Experiment II

Twelve rats were used in the course of this experiment. The rats were fasted for 12 hours and then fed a diet containing pyropheophorbide a. The amount of the sensitizer added to the diet in each instance is given in Table 3. Only the ears were examined microscopically from 7 of the rats, whereas only the eyes were examined from the other 5 rats.

The light source was the same as used for Experiment I, but the length of the exposure varied. One exposure was given in most cases.

Experiment III

Two rats were given pyropheophorbide a exactly as in Experiment I. Twenty-four hours later both rats were anesthetized with pentobarbital sodium. Only the right side of the head including the eye and ear was exposed. The parts of the body not to be exposed were screened from the light with heavy construction paper. A single exposure period of 2.5 hours was used.

RESULTS

Signs and Symptoms

The sensitized rats reacted within seconds to the light by energetic scratching and agitated movement about the exposure chamber. The excitement usually subsided after approximately 30 minutes, apparently from exhaustion. The rats developed a depressed attitude and sat with their feet and tail concealed from the light. The depression was interrupted by periods of vigorous activity throughout the remaining part of the exposure. Hyperemia and edema developed in the ears and around the eyes along with profuse lacrimation and protrusion of the third eyelid (Figures 1 and 2).

Twenty-four hours after the first exposure to light, edematous swelling of the face and head was well developed and the eyes protruded. The day following the second application of light, the normal deep red color of the eyes had faded to a pale pink. The normal color of the eye did not reappear. Opacities of the lens developed in 2 rats in Group C (rats killed 1 week postexposure) and in 3 rats of Group D (rats killed 2 weeks postexposure). Grossly visible necrosis of the skin did not occur. At the end of one week the edema of the head and ears had disappeared and only the eyes appeared abnormal.

Gross and Microscopic Findings

The gross lesions observed at the time of necropsy were the same as those seen before death. The microscopic lesions are summarized in Table 2.

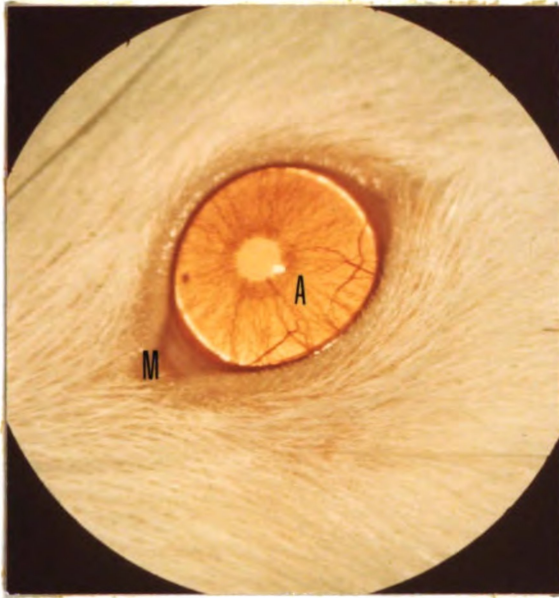


Figure 1. Eye of vehicle control rat 2 hours after initial exposure of 90 minutes to light. Experiment I, Group A. Note normal iris (A), and medial canthus (M). x 8.

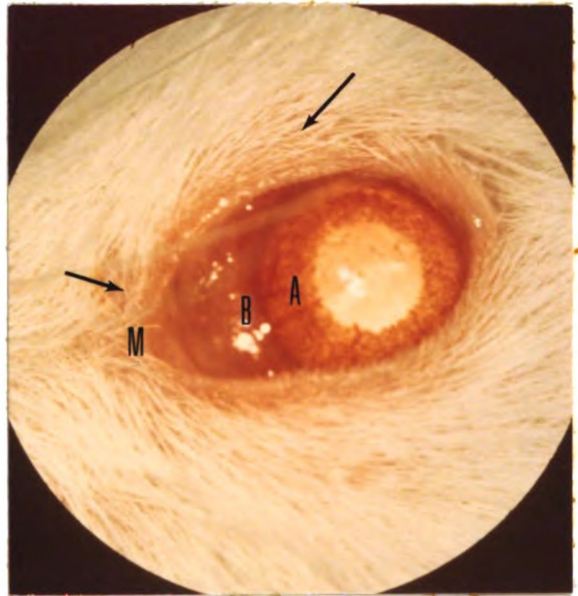


Figure 2. Eye of photosensitized rat 2 hours after initial exposure of 90 minutes to light. Experiment I, Group A. Note dilated and hyperemic iris (A), edema and protrusion of nictitating membrane (B), medial canthus (M), and lacrimal secretion on skin around eye (arrows). x 8.

Table 2. Experiment I. Comparative incidence of microscopic lesions in rats used for intravenous injection study

	Killed Post- exposure (days)	External Ear	Lacrimal Gland	Cornea	Lens	Retina
<u>Group A</u>						
VC*	0.5	0/1	1/1	0/1	0/1	0/1
DC**	0.5	0/1	0/1	0/1	0/1	0/1
T***	0.5	4/4	4/4	3/4	1/4	4/4
<u>Group B</u>						
VC	3	0/1	1/1	0/1	0/1	0/1
DC	3	0/1	0/1	0/1	0/1	0/1
T	3	4/4	4/4	0/4	0/4	4/4
<u>Group C</u>						
VC	7	0/1	1/1	0/1	0/1	0/1
DC	7	0/1	0/1	0/1	0/1	0/1
T	7	4/4	4/4	2/4	2/4	4/4
<u>Group D</u>						
VC	14	0/1	1/1	0/1	0/1	0/1
DC	14	0/1	0/1	1/1	0/1	0/1
T	14	4/4	4/4	1/4	3/4	4/4
<u>Totals</u>						
VC		0/4	4/4	0/4	0/4	0/4
DC		0/4	0/4	1/4	0/4	0/4
T		16/16	16/16	6/16	6/16	16/16

*VC = vehicle control (given vehicle and exposed to light).

**DC = dark control (sensitized with pyropheophorbide a but not exposed to light).

***T = treated rats (sensitized with pyropheophorbide a and exposed to light).

Microscopic lesions were limited to the ears and eyes and were more extensive than indicated by the clinical appearance. The greatest inflammatory response of the ears was present in rats of Group A (rats killed 12 hours postexposure). A serofibrinous exudate obliterated the normal architecture between the epithelium and the cartilage at the center of the ear and the lymphatics and veins were markedly distended. The thickness of the ear was approximately doubled. Limited numbers of neutrophils, lymphocytes and macrophages were scattered throughout the fluid exudate. The tissue of the rat's ear normally contains many mast cells. Degranulation of the mast cells was evident in sections from inflamed ears that had been stained with toluidine blue.

The ears from the rats of Groups B (rats killed 3 days postexposure), C, and D were in progressive stages of healing. In these stages the edema gradually subsided and macrophages were phagocytizing the necrotic cellular debris. The epidermis was generally thickened but occasionally there were small ulcerations infiltrated with large numbers of neutrophils and covered by scabs. Fibroblasts had proliferated and the loose subcutaneous tissue was being replaced by dense collagen fibers.

The retina was damaged in both eyes of all treated rats, whereas there were no retinal changes in either the vehicle control or dark control rats (Figure 3). In the retinas from treated rats of Group A (Figure 4) the rod cells were more basophilic than normally. They were swollen and less distinct in outline. The pigment epithelial cells were shrunken and their nuclei were pyknotic or in some instances were absent. Pyknosis, karyolysis and edema were prominent in the outer

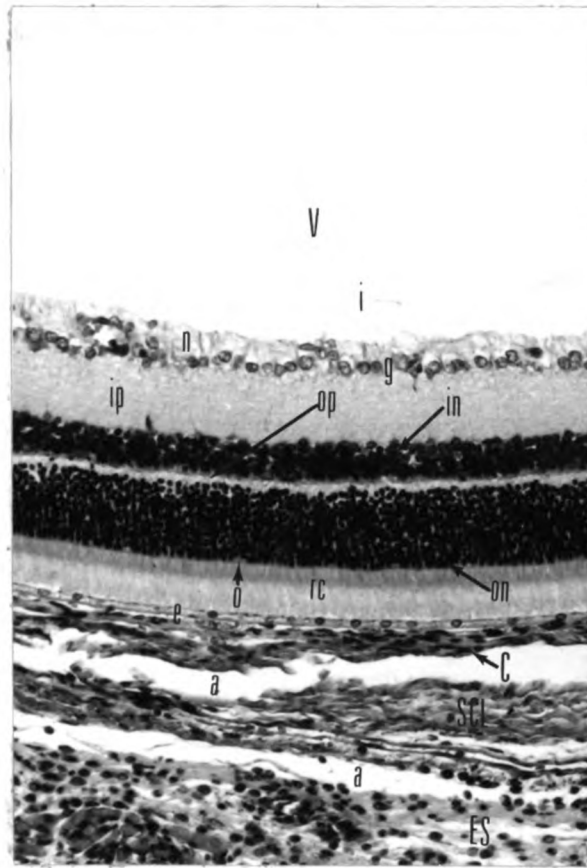


Figure 3. Normal retina and adjacent structures from vehicle control rat. Experiment I, Group A. Anatomical features are labeled: (C) Choroid, (ES) Episcleral tissue with inflammatory cells, (SCL) Sclera, (V) Vitreal space, (a) artifactual tears in ocular coats. Retinal layers from innermost to outermost: (i) inner limiting membrane - has been pulled from surface of retina in processing, (n) nerve fiber layer, (g) ganglion cell layer, (ip) inner plexiform layer, (in) inner nuclear layer, (op) outer plexiform layer, (on) outer nuclear layer, (o) outer limiting membrane - hardly visible, (rc) rod and cone cell layer, and (e) pigment epithelium - pigment lacking in albino rat. Zenker's fixation. Hematoxylin and eosin. x 187.

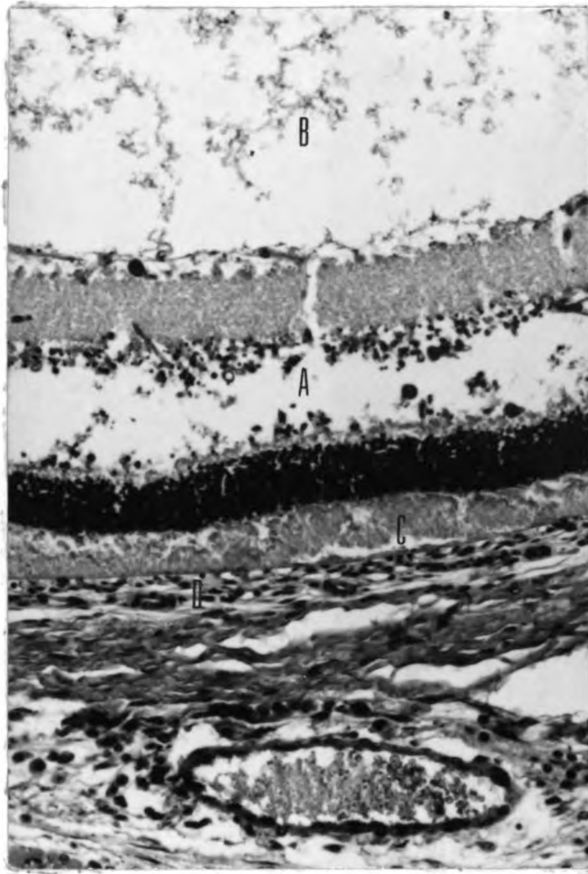


Figure 4. Early retinal degeneration in eye from photosensitized rat. Experiment I, Group A. Note involvement of all retinal layers with prominent edema and necrosis of inner nuclear layer (A), precipitated material in vitreal space (B), and fragmentation of rod and cone cells (C) (some of the latter is artifact). The choroid (D) is nearly normal. Zenker's fixation. Hematoxylin and eosin. x 187.

nuclear layer. Changes in rod cell nuclei of the latter layer were easier to detect in the Zenker fixed specimens than in those fixed in formalin. The rod cell nuclei had a rather uniformly irregular outline that was readily observable in properly fixed specimens. Assumption of a smooth and rounded outline was the first observable response when the rod cell nuclei were injured. Serous exudate accumulated in the outer plexiform and inner nuclear layers. Changes in the other retinal layers were not detectable at this stage.

The retinas from rats in Group B (Figures 5 and 6) were extremely edematous. The fluid accumulated within the layers of the retina and resulted in retinoschisis. The rent developed either in the outer nuclear, the outer plexiform or the inner nuclear layer. The retinal layers outside of the inner nuclear layer had mostly disappeared by this time and large mononuclear phagocytes had infiltrated the area. In some instances small, well defined granulomas had formed. Other types of inflammatory cells were rare.

The retinas from rats in Groups C (Figure 7) and D (Figures 8 and 9) were similarly affected. Characteristically, all that remained of the retina was one or two fine strands of connective tissue. A small amount of recognizable retinal tissue composed of the four inner retinal layers was present near the ora serrata in some cases.

Occasionally, mineral deposits surrounded by a few macrophages were closely adherent to the choroid (Figure 8). Otherwise the macrophages had almost completely disappeared. The mineral stained positively for calcium by Kossa's method and with alizarin red. Gomori's method for staining iron was negative.

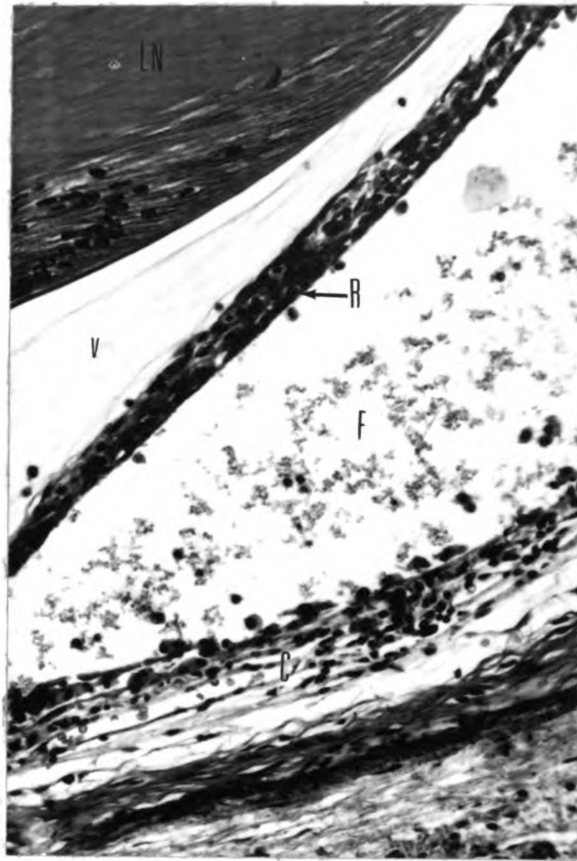


Figure 5. Section through eye of photosensitized rat to show accumulation of fluid in retinal defect with most of vitreal substance displaced. Experiment I, Group B. Note fluid filled space (F) that elevates retinal remnant (R), and inflammatory cells in choroid (C). (V) is vitreal space, (LN) is part of normal lens. Zenker's fixation. Hematoxylin and eosin stain. x 187.

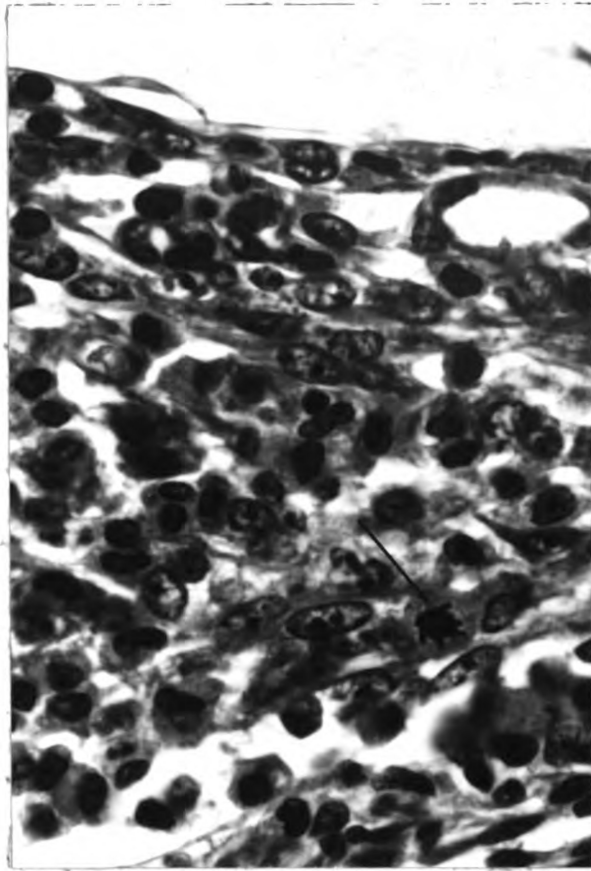


Figure 6. Granulomatous reaction in retina of photosensitized rat. Experiment I, Group B. Note mitotic figure of proliferating macrophage (arrow). Zenker's fixation. Hematoxylin and eosin. x 750.

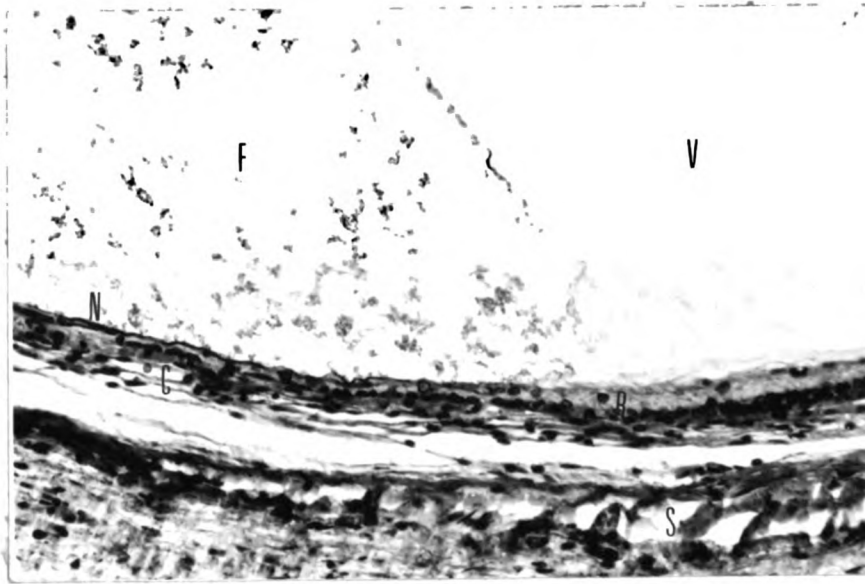


Figure 7. Nearly complete retinal degeneration in eye of photosensitized rat. Experiment I, Group C. Note remnant of inner retinal layers (R), increased staining of vitreal substance (V) - (compare with Figure 1), fluid accumulation which has displaced part of vitreal substance (F), area with no recognizable retina (N), slight infiltration of choroid (C) with inflammatory cells, and distorted sclera (S) which is artifact. Zenker's fixation. Hematoxylin and eosin. x 187.

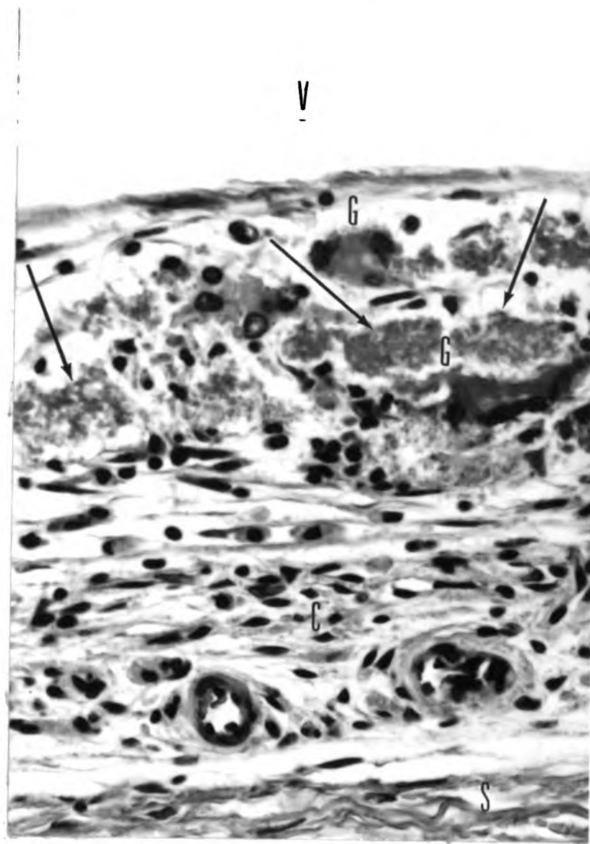


Figure 8. Degenerate retina of photosensitized rat with mineralized cellular debris. Experiment I, Group D. Note accumulation of mineral (arrows), giant cells (G), the choroid (C), vitreal space (V), and part of sclera (S). Zenker's fixation. Hematoxylin and eosin. x 468.

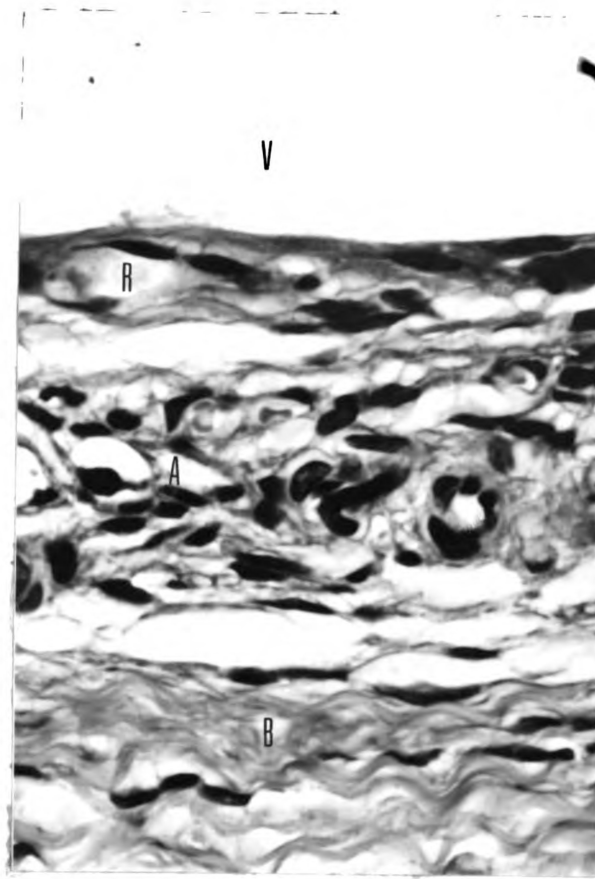


Figure 9. Degenerate retina from photosensitized rat. Experiment I, Group D. Note the strands of connective tissue that represent what remains of the retina (R), the choroid (A), the sclera (B), and the vitreal space (V). Zenker's fixation. Hematoxylin and eosin. x 750.

The choroid was only slightly affected at the earliest stage of the retinal degeneration but was later infiltrated with a mixture of inflammatory cells. The layers of the choroid of rats in Group D had spread apart somewhat and the number of capillaries was reduced.

The iris was not often, if ever, damaged. The aqueous humor usually contained an increased amount of proteinaceous material that precipitated as pink staining granular strands or clumps. Some fibrin was present.

The vitreous humor contained a variable quantity of fibrin. Fluid filled dilatations of the retina often extended to the posterior surface of the lens, thereby occupying much of the vitreous space.

The incidence of lenticular lesions is summarized in Table 2. The identification of early lenticular changes in the eyes was difficult; however, well developed cataracts were easily distinguished. Formalin-fixed specimens were best suited for study of the lens. The cataracts observed grossly in the rats before death were easily identified microscopically. The cataractous changes were limited to the subcapsular region of the affected lenses. The cataracts were bilateral in all instances except one. All of the rats that had cataracts were in the treated groups.

Corneal lesions occurred in 7 of the 24 rats as indicated in Table 2. All except one of the rats with corneal lesions were in the treated group. The lone exception was a dark control. Keratitis and superficial ulcerations of the corneal epithelium were the only changes observed. Grossly recognizable pus was noted clinically in 3 of the 4 rats from Groups C and D that had corneal lesions.

There was extensive damage to the lacrimal glands of all rats that were exposed to light. The changes were the same in the glands of both the vehicle control and treated rats. No damage occurred in the lacrimal glands of dark control rats. The damaged portion of the glands was located adjacent to the posterior aspect of the ocular globe and extended approximately half the thickness of the gland.

Initially, the glandular epithelial cells were lysed and remained in the acini as a foamy, granular mass containing remnants of pyknotic nuclei (Figures 10 and 11). The connective tissue framework extending into and around the gland was distended with serofibrinous exudate.

Later, the inflammatory reaction was of the granulomatous type with large numbers of foamy macrophages that had engulfed the necrotic cellular debris. Areas of mineralized necrotic material surrounded by multinucleated giant cells were present in most cases. Squamous metaplasia of the glandular epithelium occurred with regularity.

Regeneration of normal glandular tissue had begun in the glands harvested 2 weeks after exposure.

The data of Experiment II show that pyropheophorbide a can photosensitize rats when ingested with the diet. The microscopic observations are summarized in Table 3.

The microscopic changes of the eyes and ears were generally similar to those described for Experiment I except in rat No. 12. This rat died 20 minutes following a 2.5 hours exposure to light. The retinal changes were limited to a loss of the normal staining properties of the rod cells and pyknosis of the nuclei in the outer nuclear layer. There were acute necrotic changes in the lacrimal glands with serous exudation.

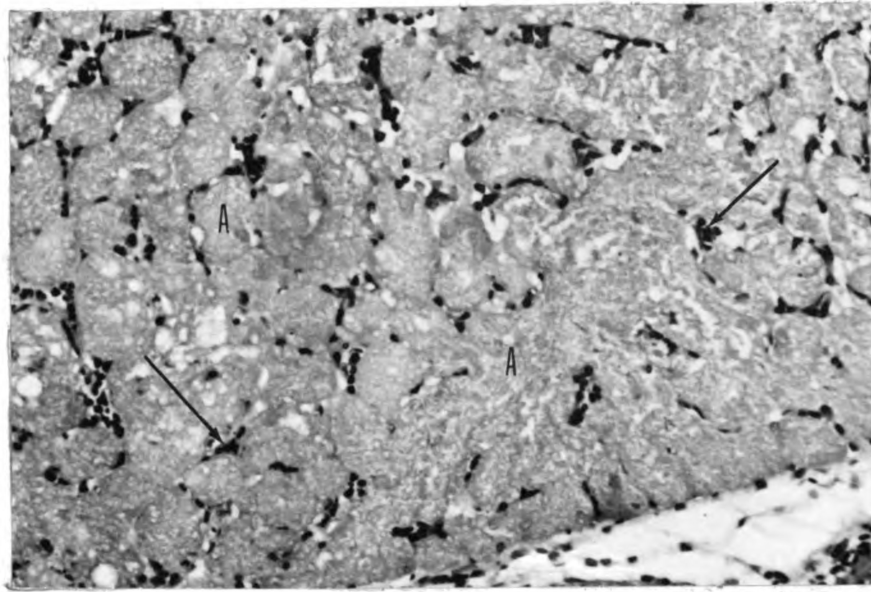


Figure 10. Acute coagulative necrosis of lacrimal gland from photosensitized rat. Experiment I, Group A. Note complete absence of recognizable glandular epithelial cells and granular material filling the glandular spaces (A), also the pyknotic nuclei (arrows). Compare with Figure 11. Formalin fixation. Hematoxylin and eosin. x 187.

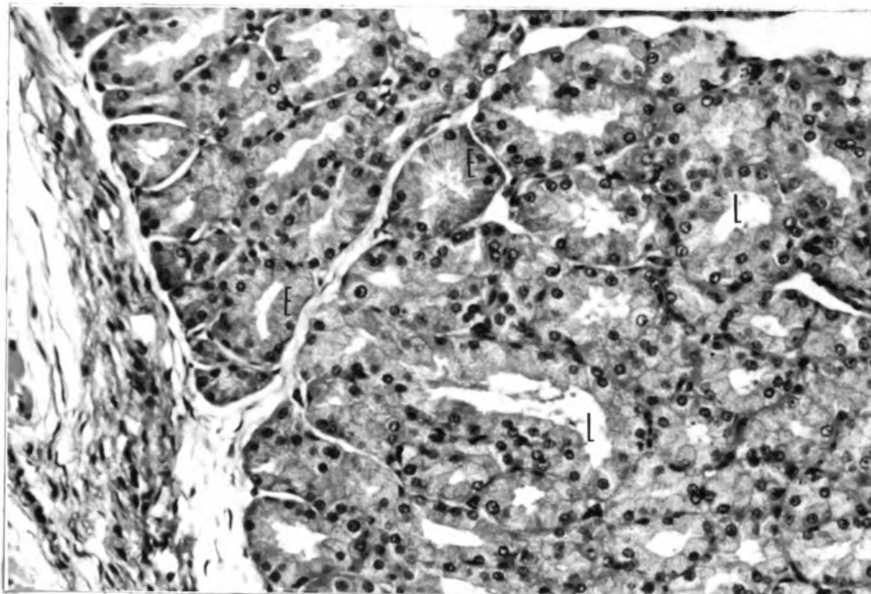


Figure 11. Normal lacrimal gland from dark control rat. Experiment I, Group B. Note normal epithelial cells (E) and the open lumen of some acini (L). Formalin fixation. Hematoxylin and eosin. x 187.

Table 3. Experiment II. Location of microscopic lesions in rats sensitized by feeding pyropheophorbide a

Rat No.	Dose (mg.)	Structures Examined				
		External Ear	Lacrimal Gland	Cornea	Lens	Retina
1	none	0*	N**	N	N	N
2	none	0	N	N	N	N
3	none	N	+***	0	0	0
4	4	N	+	0	0	0
5	7	N	+	0	0	+
6	10	+	N	N	N	N
7	10	+	N	N	N	N
8	10	+	N	N	N	N
9	10	+	N	N	N	N
10	10	N	+	0	0	+
11	10	+	N	N	N	N
12	15	N	+	0	0	+

*0 = lesions not present

**N = not examined

***+ = lesions present

Rat No. 4 did not have retinal changes. It was concluded that the amount of sensitizer ingested (4 mg.) was insufficient to cause photosensitivity.

One of the 2 rats in Experiment III died shortly after the exposure was finished. The exposed (right) eye had microscopic changes similar to those described for rat No. 12 of Experiment II. The retina and lacrimal gland of the unexposed (left) eye were both completely normal.

The second rat of this experiment survived the exposure and was killed 7 days later. A severe lesion developed which was sharply limited to the area of the head and face that had been exposed. Initially, there was a pronounced edema of the exposed area which reached its maximum extent 24 hours after exposure. A dry fibrinonecrotic membrane formed at the surface of the cornea (Figure 12).

Subsequently, the tissues became discolored proceeding from purple, to blue, to blue-black. The external ear became dry and wrinkled. Much of the hair was lost from the skin in the exposed area.

Microscopically, the external ear was a typical example of dry gangrene. The skin covering the side of the face was necrotic with an abundant infiltration of neutrophils. The necrosis extended deeply and involved the muscles on the side of the face. The cornea had ruptured and the ocular globe was collapsed. A primarily neutrophilic inflammatory process extended to all portions of the orbit.

In contrast to the exposed (right) side of the face, the unexposed (left) side of the face was not affected. The external ear, the retina, and the lacrimal gland were histologically normal (Figure 13).



Figure 12. View from right side of photosensitive rat from Experiment III 6 days after right side of head was exposed to light. Note shriveled ear, scab encrusted cornea and the loss of hair around the eye. Approximately life size.



Figure 13. Left (unexposed) side of rat in Figure 12 and photographed on the same day. Note normal appearance of eye and ear. Approximately life size.

DISCUSSION AND CONCLUSIONS

The general syndrome of photosensitization observed in the rats of this study was similar to that reported by Mathews (1938). The appearance was almost identical with respect to the clinical picture and the microscopic changes in sections of the ear and cornea. From these results it is clear that the rats were indeed photosensitized by intravenous injections (Experiments I and III) and ingestion of (Experiment II) pyropheophorbide a.

Lesions of the intraocular tissues and lacrimal glands were not reported by Mathews (1938). In reviewing the literature several reports were found in which intraocular lesions were associated with photosensitization or photosensitizing agents. A direct cause and effect relationship between the intraocular lesions and a photodynamic agent was not established in any of these reports.

Retinal damage was observed in the examined eyes of all rats (the eyes were not examined from 7 of the 12 rats of Experiment II) that had been given pyropheophorbide a and that were subsequently exposed to light except rat No. 4 of Experiment II. This rat was fed 4 mg. of pyropheophorbide a which was an insufficient amount to photosensitize a rat. Clare (1953) and Tsutsumi and Hashimoto (1964) reported that 7 mg. was the minimal oral dose for sensitizing rats. The results of Experiment II agree with these earlier reports.

The absence of retinal damage in the dark control and vehicle control rats of Experiments I and II and in the unexposed eyes of the 2

rats in Experiment III is evidence that retinal damage was not due to the vehicle, the light or the pyropheophorbide a alone.

The retinal changes that Noell *et al.* (1966) produced in rats with light in the absence of a photodynamic agent differed in several important respects from the changes observed in this study. They reported that 24 hours of continuous exposure to light was required for irreversible retinal changes when the rats were exposed at normal body temperature. The histologic lesions under these conditions were not present until 4 to 6 weeks after exposure. Regardless of the method they used to expose the rats, the retina was never completely destroyed. Portions of the inner nuclear layer and the layers internal to the inner nuclear layer always remained. The histologic changes reported by Dantzker and Gerstein (1969) were the same as those reported by Noell *et al.* (1966).

The light used by Noell *et al.* (1966) and Dantzker and Gerstein (1969) was less intense than that used in this study. The absence of lesions in the eyes of vehicle control rats, which were exposed to light, tends to refute a suggestion that the more severe retinal lesions of the present study were due to the greater intensity of light.

The report by Pierpaoli and Santamaria (1961) of light induced damage to isolated calf's retina tends to support the work of Noell *et al.* (1966) and Dantzker and Gerstein (1969) but is not helpful in understanding the retinal changes observed in the present study. The retinal lesions reported by Walkowicz (1956) are difficult to interpret. He used rabbits which had been given 10 mg. of eosin 7 days prior to exposure. Since eosin is rapidly excreted, it would be interesting to

know the amount that remained in the body after 7 days. The light source he used was a highly focused narrow beam. Possibly the retinal changes were due to heat.

The reports of Jaffe (1950) and Barnes and Boshoff (1952) of retinal lesions in porphyric patients were clinical reports without histologic confirmation. These reports indicate a need for further studies of retinal damage caused by photodynamic action.

The explanation as to why retinal damage occurred in the rats of this study but has not been reported in the many other photosensitizations of rats and other species can only be conjectural. The following are possible explanations:

1. The eye is often disregarded by pathologists and thus retinal lesions may have been overlooked.
2. The photodynamic agent located in the deeper tissues of the eye was not excited because sufficient energy at the required wavelength could not reach it. The various photodynamic agents must absorb radiant energy of specific wavelengths to initiate the damaging oxidative reactions. The wavelengths that excite the different photosensitizers lie within the near ultraviolet ($\lambda > 320$ nm.) and visible portions of the spectrum. The penetration of tissues by these radiations varies considerably.
3. The photodynamic agent was not distributed to the necessary location within the ocular tissues to receive the radiant energy.
4. The retina of the albino rat may be unusually susceptible to photodynamic damage because the lack of pigment permits greater penetration of light. The retinal lesions must have been overlooked in earlier studies if this alternative is correct.

5. The retinal damage observed in this study was not due to photodynamic action. This alternative cannot be entirely eliminated using the evidence at hand.

The earliest changes within the retina were not precisely localized. The first changes observed, however, were in the rod cells and the rod cell nuclei. After the initial injury the progress of the degenerative changes was very rapid; thus a meaningful study of the progressive cellular changes was impossible. A series of rats killed at very short intervals following exposure would be required to determine the sequence of changes.

The lesions of the lacrimal gland occurred in all rats that were exposed to light without regard to other factors. The lacrimal glands of rats not exposed to light were normal. The explanation of these lesions of the lacrimal gland is conjectural; however, it seems certain that the changes were not directly related to photodynamic action mediated by pyropheophorbide a. Prince (1964) reported that the lacrimal gland of the rat contains free porphyrin compounds and that the gland fluoresces under ultraviolet light. It is possible that the intense light used for the present experiments was able to penetrate through the posterior wall of the ocular globe and into the tissue of the lacrimal gland directly beyond. The natural porphyrin compounds of the lacrimal gland could have mediated the photodynamic destruction of the glandular tissue. The histologic changes in and around the lacrimal gland were consistent with a photosensitization. Reports could not be found in the literature to support this hypothesis.

The lenticular changes observed in the rats of this study were somewhat inconsistent but the incidence increased as the length of

time after exposure increased. The most likely explanation is that the degeneration of the lens occurred secondarily to the damage of the retina and choroid. The increased intraocular pressure caused by the exudation of fibrin would disturb circulation of the aqueous humor and result in impaired provision of nutrients to the lens. Degeneration of the lens is rapid under these conditions. There was no evidence to associate the changes in the lens to photodynamic action or to the direct action of light.

SUMMARY

Albino rats were photosensitized by ingestion or by intravenous injection of pyropheophorbide a, a chlorophyll derivative, and subsequent exposure to an artificial source of light.

Suitable controls for the effects of the vehicle and of the sensitizer when the animal is not exposed to light were included in the study.

The typical lesions of photosensitization were produced in rats sensitized by either route of administration. In addition, lesions occurred in the intraocular tissues and the lacrimal glands. Severe retinal damage due to photodynamic action was a constant finding that has not been reported previously.

The lacrimal glands were damaged in all rats exposed to light whether sensitized to it or not. A natural photodynamic mechanism was postulated as the cause of lesions of the lacrimal glands.

Cataracts developed in 6 of 16 rats that were sensitized to light by intravenous injection of pyropheophorbide a. The cataracts were interpreted as secondary changes resulting from photodynamic lesions in the retina and choroid.

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